# Phenotypic analysis of osteoclast lineage in c-fos mutant mice

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# Phenotypic analysis of the osteoclast lineage in *c-fos* mutant mice

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# DEDICATION

**To my parents and my sister**

**for their support**

# DECLARATION

**No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.**

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# Abstract

Osteoclastogenesis is closely controlled by local and systemic factors, and transcriptional regulators. Previous results have shown that mice lacking c-Fos develop osteopetrosis due to a complete block in osteoclast differentiation, whereas mice overexpressing c-Fos develop remodelling osteosarcomas containing numerous osteoclasts. The aims of this project were to understand further the function of *c-fos* on osteoclast differentiation and function both *in vivo* and *in vitro*.

The expression of osteoclast, macrophage and osteoblast marker genes were first mapped in both c-Fos knockout bones as well as in c-Fos transgenic osteosarcomas, in order to investigate the consequences of altered c-Fos levels on these cell types *in vivo*. The osteoclastic phenotypes in *c-fos* knockout mice suggest that in the cascade of osteoclast differentiation, *c-fos* is downstream of PU.1, *c-fms* and RANK, upstream of *mi*, and in a similar position to *NFκB*. In c-Fos transgenic osteosarcomas, a large number of multinucleated and mononuclear cells with osteoclast and macrophage phenotypes are present not only within neoplastic bone areas but also in the fibrous tumour margins not containing any bone. The strong expression of osteoblastic marker genes and osteoclast cytokines may partially account for the ectopic osteoclastic cells.

To further investigate the effects of *c-fos* on osteoclast precursors, *in vitro* studies were performed using primary M-CSF-dependent bone marrow and spleen cells derived from *c-fos* knockout mice and transgenic mice. Surprisingly, functional TRAP positive multinucleated cells were formed from newborn bone marrow cells lacking c-Fos, but not from mutant spleen cells, and this osteoclastic potential in c-Fos knockout precursors was lost with increasing age. Semi-quantitative RT-PCR analysis showed that in younger animals, upregulation of *fra-1* may compensate for the deficiency of c-Fos. Addition of growth factors, such as TGF-β and TNF-α could not fully substitute for the absence of c-Fos in osteoclastogenesis. Bone marrow precursors expressing exogenous *c-fos* exhibited an increased responsiveness to RANKL and M-CSF signalling, which resulted in enhanced osteoclast formation and resorption pit formation. The data suggested that these effects may be mediated by

molecules downstream of RANKL/RANK signalling, such as TRAF-6, *fra*-1 and IFN-β.

Taken together, these data provide novel information on the role of c-Fos in osteoclast differentiation which may challenge the current published theories.

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# Chapter 1

# Introduction

## 1.1 Bone structure

Bone is a specialised form of connective tissue. It provides structural support for the body in all higher vertebrates, protects delicate structures and maintains calcium homeostasis (Watts, 1999). Morphologically there are two forms of bones: flat bones (*e.g.* bones in the skull) and long bones (*e.g.* tibia, femur, humerus). Long bones have epiphyses on both ends, a diaphysis in the middle and a metaphysis in between. In growing long bones, the epiphysis and metaphysis are separated by a layer of cartilage, called the growth plate, which is responsible for longitudinal growth and which becomes calcified and replaced by bone in mature long bones. Mature bones consist of dense surface plates of calcified tissues, the cortex, within which is a network of thin calcified trabeculae that are oriented along lines of forces. In the diaphysis, a hollow marrow space full of haematopoietic cells and adipose tissues is enclosed by cortex (Blair, 1998) (Fig.1.1). As in all connective tissue, bone is composed of cells and extracellular matrix.

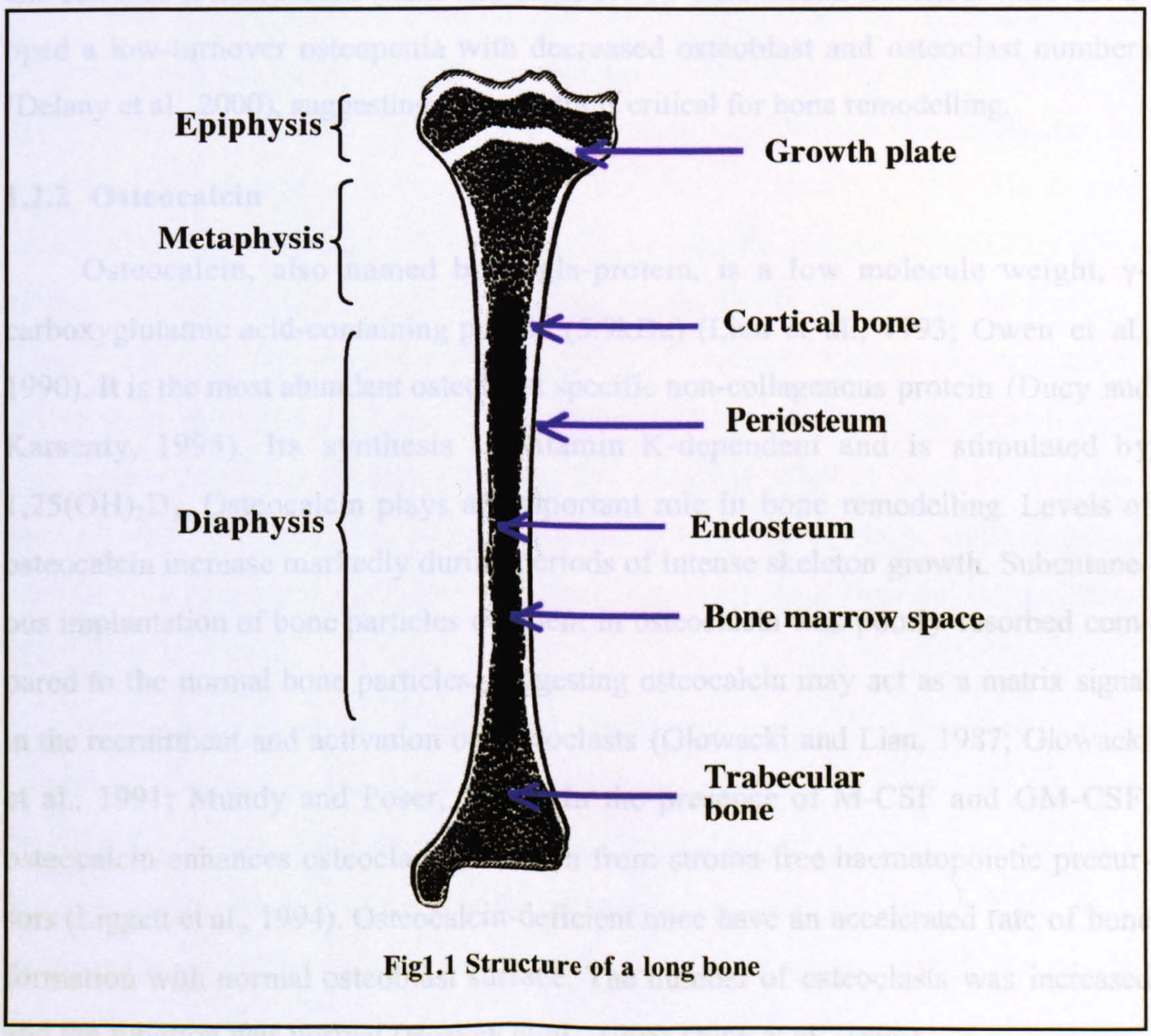



**Fig1.1 Structure of a long bone**

## 1.2 Extracellular matrix

Bone matrix consists of both organic and inorganic components. The main components of the inorganic bone are calcium phosphate in the form of calcium hydroxyapatite [(HA), $Ca_{10}(PO_4)_6(OH)_2$]. Small quantities of sodium, magnesium and fluoride are present in the bone in association with HA. 90% of the total organic bone matrix is type I collagen secreted by osteoblasts (Marks and Popoff, 1988). Type I collagen is oriented along lines of stress, which resists compression. The remainder of the organic phase consists of non-collagenous proteins, as described below.

### 1.2.1 Osteonectin

The most abundant non-collagenous protein is osteonectin. Osteonectin, also known as SPARC, is a 43kDa acidic phosphorylated glycoprotein, which is synthesised by osteoblasts and odontoblasts (Young et al., 1992). *In vitro* studies indicated that osteonectin binds collagen and hydroxyapatite and can regulate cell proliferation and cell-matrix interactions (Lane and Sage, 1994). Osteonectin knockout mice developed a low-turnover osteopenia with decreased osteoblast and osteoclast numbers (Delany et al., 2000), suggesting osteonectin is critical for bone remodelling.

### 1.2.2 Osteocalcin

Osteocalcin, also named bone gla-protein, is a low molecule weight, γ-carboxyglutamic acid-containing protein (5.9kDa) (Lian et al., 1993; Owen et al., 1990). It is the most abundant osteoblast specific non-collagenous protein (Ducy and Karsenty, 1995). Its synthesis is vitamin K-dependent and is stimulated by $1,25(OH)_2D_3$. Osteocalcin plays an important role in bone remodelling. Levels of osteocalcin increase markedly during periods of intense skeleton growth. Subcutaneous implantation of bone particles deficient in osteocalcin was poorly resorbed compared to the normal bone particles, suggesting osteocalcin may act as a matrix signal in the recruitment and activation of osteoclasts (Glowacki and Lian, 1987; Glowacki et al., 1991; Mundy and Poser, 1983). In the presence of M-CSF and GM-CSF, osteocalcin enhances osteoclast formation from stroma-free haematopoietic precursors (Liggett et al., 1994). Osteocalcin-deficient mice have an accelerated rate of bone formation with normal osteoblast surface. The number of osteoclasts was increased and the function was normal (Boskey et al., 1998; Ducy et al., 1996).

### 1.2.3 Osteopontin

Osteopontin (OPN) is a 32kDa phosphorylated calcium binding protein. This protein contains RGD (Arg-Gly-Asp) integrin-binding sequence that interacts with integrins of the $\alpha_v$ class (Ross et al., 1993). Osteopontin has been shown to be a potentially important protein in bone remodelling (Terai et al., 1999; Yamazaki et al., 1999). It is expressed during bone development at sites of remodelling. The attachment of osteoclasts during bone resorption depends upon the interaction between osteopontin and the integrin $\alpha_v\beta_3$ (Miyauchi et al., 1991; Ross et al., 1993). Antibodies against osteopontin and $\alpha_v\beta_3$ inhibited the attachment of osteoclasts to the intact bone matrix. Osteoclast $\alpha_v\beta_3$ and bone matrix osteopontin colocalise. In murine osteoclasts, osteopontin localizes to the clear zone and ruffled border membranes. It is secreted into the resorption pits during bone resorption, where it binds to $\alpha_v\beta_3$ and stimulates osteoclast migration by cytoskeletal rearrangement (Chellaiah and Hruska, 2003; Reinholt et al., 1999). Osteopontin-deficient (*OPN*-/-) mice generated by targeted disruption of *spp1* (secreted phosphoprotein 1) gene showed normal bone development and structure, but with an increased trabecular bone area in *OPN*-/- femurs. Osteoclasts isolated from *OPN*-/- mice were significantly less motile and exhibited decreased bone resorptive activity (Chellaiah and Hruska, 2003). The number of osteoclasts formed from *OPN* knockout haematopoietic precursors in the coculture system was significantly increased (Rittling et al., 1998), which may be due to a compensation for the reduced bone resorption. Exogenous osteopontin rescued the defect in motility, but only partially rescued the function suggesting deposition of osteopontin into the resorption pits from the osteoclast itself enhanced osteoclastic bone resorption. Osteopontin also plays a role in osteoclastogenesis, since antiserum specific to osteopontin inhibited osteoclast formation from bone marrow cultures (Yamate et al., 1997).

Osteopontin is also involved in bone resorption induced by hormonal and mechanical changes. It was reported that *OPN* deficiency prevent bone loss following ovariectomy in mice (Yoshitake et al., 1999). Furthermore, *OPN* knockout mice were resistant to bone resorption induced by mechanical unloading (Ishijima et al., 2001). In the unloaded *OPN* knockout mice, osteoclast number was decreased compared to the wild-type, but *in vitro*, RANKL-induced osteoclast formation was similar, sug-

gesting the mechanisms by which osteopontin regulates osteoclastogenesis might involve the expression of RANKL.

### 1.2.4 Bone sialoprotein

Bone sialoprotein (BSP) is an acidic bone phosphoprotein. It is highly expressed in mature osteoblasts, but not in immature precursors (Bianco et al., 1991; Chen et al., 1993). It has been reported that BSP is involved in extracellular matrix mineralisation (Hunter and Goldberg, 1993). In addition, BSP, Like OPN, contains an RGD (Arg-Gly-Asp) integrin-binding sequence, suggesting it may play a role in osteoclast adherence to bone similar to osteopontin. In fact, RGD-containing peptides derived from BSP inhibited the attachment of osteoclasts to bone matrix (Ross et al., 1993).

Taken together, although bone matrix proteins are secreted by osteoblasts, they are important for osteoclast formation, recruitment and function, which suggests that during bone remodelling, bone formation and bone resorption are tightly coupled together.

## 1.3 Bone cells

The cells in bone are generally subdivided into (1) osteoblasts (bone forming cells), (2) osteocytes and bone lining cells and (3) osteoclasts (bone resorbing cells).

### 1.3.1 The osteoblast phenotype

Osteoblasts are cuboidal cells situated along the bone surface, which are responsible for the production of the matrix constituents (collagen and ground substance). They also play an essential role in regulating osteoclast differentiation and activation by producing factors (Hofbauer et al., 2000) (see also section 1.5.2-4). Osteoblasts have a round nucleus at the base of the cell and a strongly basophilic cytoplasm (Holtrop, 1975). The plasma membrane of the osteoblast is rich in alkaline phosphatase, and has receptors for many osteotropic hormones, including parathyroid hormone, estrogen and vitamin $D_3$ (Bodine et al., 1998; Li et al., 1997). Alkaline phosphatase (ALP) is used as a marker of osteoblastic development (Stein and Lian, 1993). The function of ALP has been demonstrated by gene inactivation studies: Tissue non-specific alkaline phosphatase (*TNAP*) knockout mice appeared normal at

birth, but die of epileptic seizures before weaning. Osteoblasts isolated from *TNAP* -/- mice differentiated normally and were able to form cellular nodules *in vitro*, but were unable to mineralise these nodules. *TNAP*+/- osteoblasts also showed delayed mineralisation. Differentiation and function of osteoclasts were unaffected by the deletion of *TNAP* (Wennberg et al., 2000).

### 1.3.1.1 Osteoblast differentiation

Osteoblasts originate from local mesenchymal stem cells (bone marrow stromal stem cell or connective tissue mesenchymal stem cells), which also give rise to chondrocytes, fibroblasts, myoblasts, and adipocytes (Fig.1.2). These precursors, with the right stimulation, undergo proliferation and differentiate into preosteoblasts and then into mature osteoblasts. It has been reported that osteoblasts undergo a three-stage process of differentiation: proliferation, matrix maturation and mineralisation (Aubin et al., 1995; Owen et al., 1990). A well-characterized temporal and spatial expression pattern of extracellular bone matrix proteins and other genes occurs in this process. During proliferation period, growth related genes and genes associated with formation of the extracellular matrix (ECM) are actively expressed, including type I collagen, fibronectin and TGF-β. After the downregulation of proliferation, proteins associated with the osteoblast phenotype are detected, such as ALP, PTH/PTHrP receptor, bone sialoprotein and osteocalcin. *ALP* gene is maximally expressed at this stage. Finally, the ECM is calcified and the level of *ALP* is downregulated (Stein and Lian, 1993). Fig.1.3 shows the genes involved in osteoblast and chondrocyte differentiation (Adapted from Aubin et al., 1995).

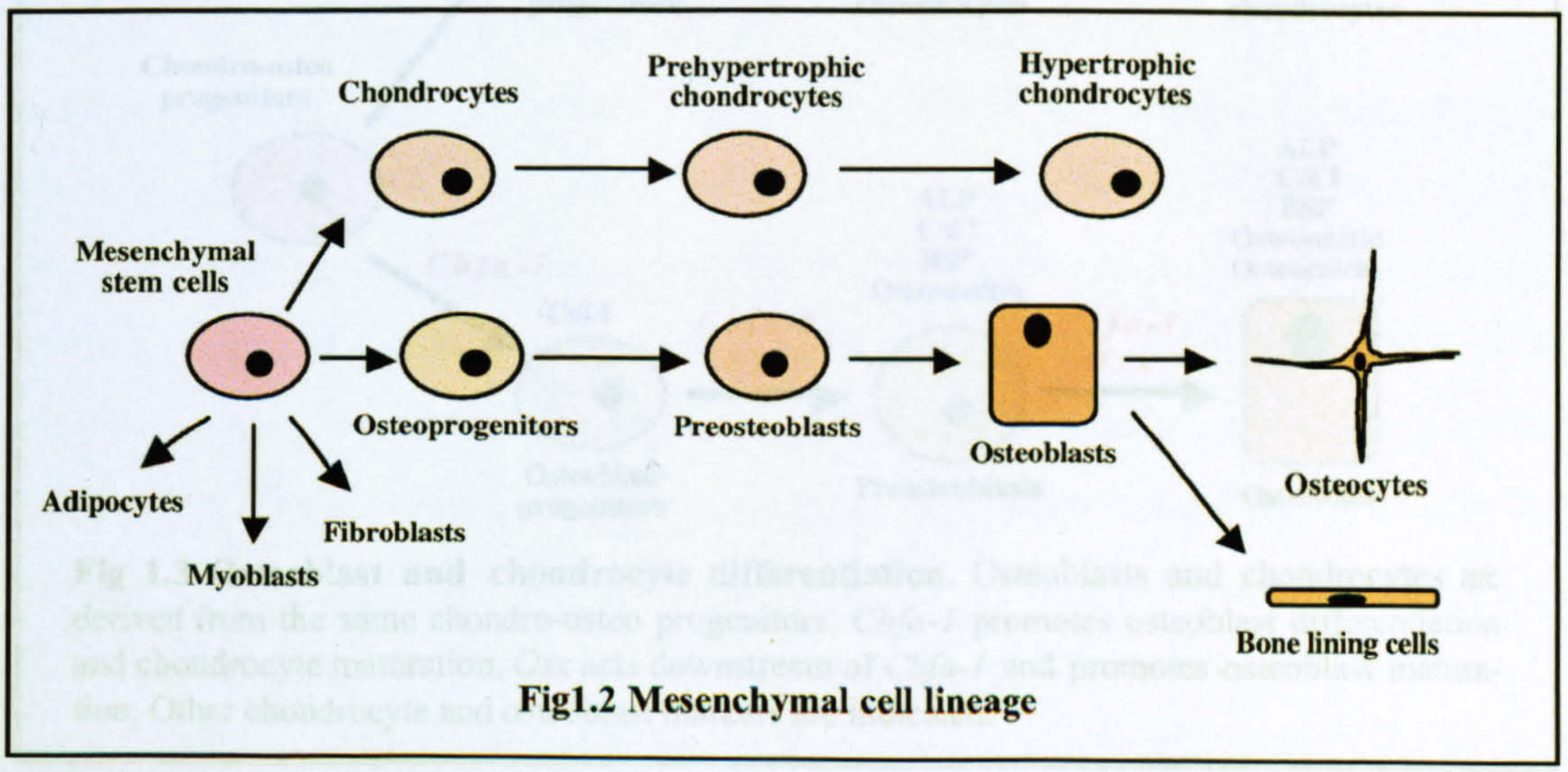



**Fig1.2 Mesenchymal cell lineage**

### 1.3.1.2 Role of *Cbfa-1* in osteogenesis

To date, only two genes are known to control osteoblast differentiation directly or indirectly: *Cbfa-1* and *Indian hedgehog (Ihh)* (Karsenty, 1999). In endochondral ossification during embryogenesis, the mesenchymal progenitors grouped in skeletal condensations first differentiate into type II collagen producing cells. *Cbfa-1* then promotes their differentiation into *Ihh*-secreting hypertrophic chondrocytes or into type I collagen-producing preosteoblasts. In addition to regulating nonhypertrophic chondrocyte proliferation, *Ihh* also affects osteoblast differentiation. Mice deficient in *Ihh* have a disorganized growth plate and no osteoblasts present in the mutant long bones (St-Jacques et al., 1999), suggesting that during endochondral ossification, *Cbfa-1* is upstream of *Ihh* in the control of chondrogenesis but downstream in the control of osteogenesis.

*Cbfa-1* (core-binding factor)/ *Runx2* (runt-related gene 2), also called *osf2* (osteoblast-specific transcription factor 2) is a transcription factor of the *runt* domain gene family that is essential for bone formation during embryogenesis. During skeletal development, *Cbfa-1* is expressed exclusively in mesenchymal cell condensation at early stages (12.5 dpc), and the expression is restricted to osteoblast lineage thereafter and turned off in differentiated chondrocytes (Fig.1.3). It has been used as the earliest and most specific marker gene for osteogenesis.

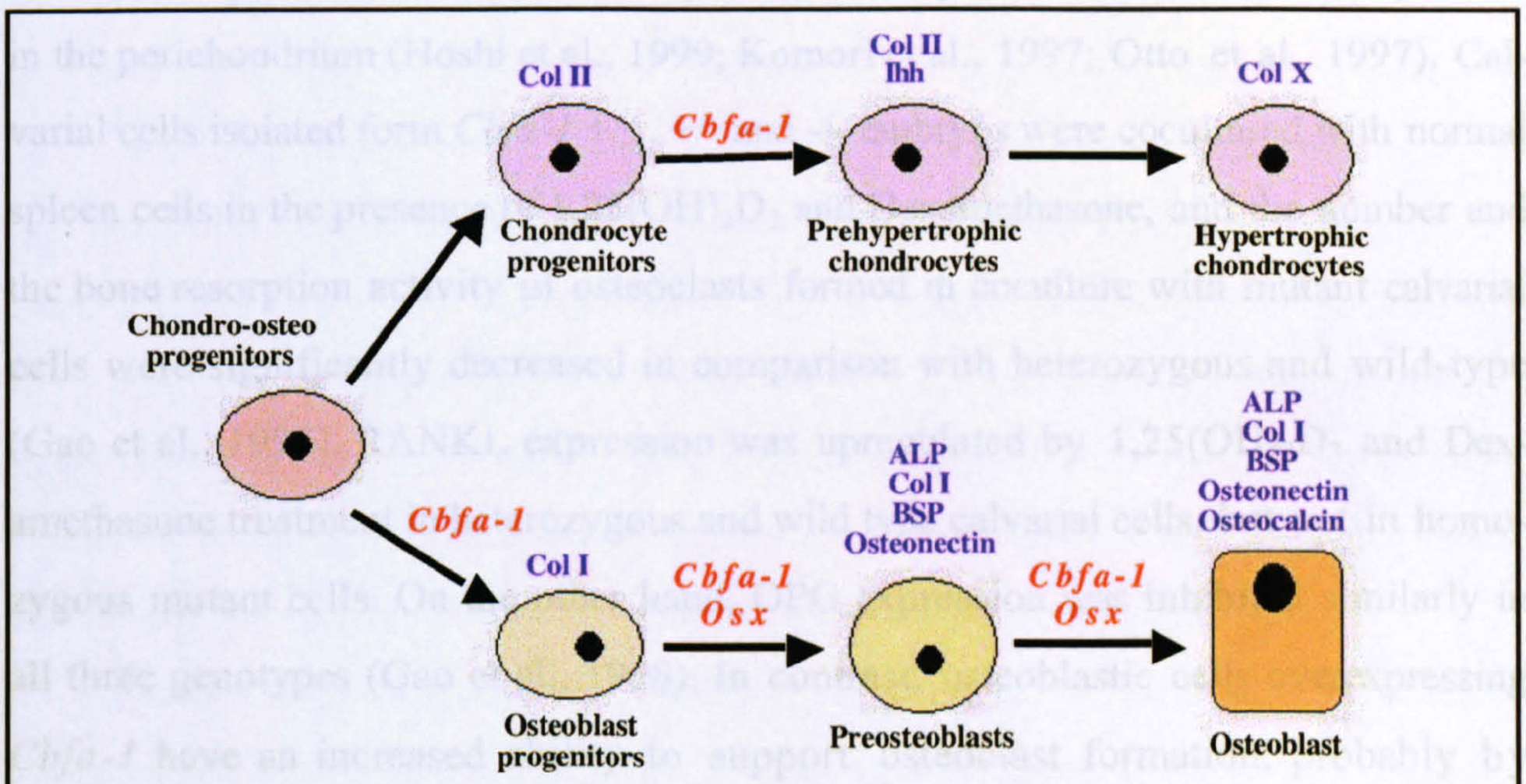



**Fig 1.3 Osteoblast and chondrocyte differentiation.** Osteoblasts and chondrocytes are derived from the same chondro-osteo progenitors. *Cbfa-1* promotes osteoblast differentiation and chondrocyte maturation. *Osx* acts downstream of *Cbfa-1* and promotes osteoblast maturation. Other chondrocyte and osteoblast markers are indicated.

*Cbfa-1* is critical for osteoblast development. *Cbfa-1* deficient mice developed a cartilaginous skeleton. Both intramembranous and endochondral ossification were completely suppressed because of the lack of osteoblast differentiation. These mice also showed defects in chondrocyte maturation, which was arrested at a stage before hypertrophy. In *Cbfa-1* +/- mice, the defects were confined to intramembranous bone formation and the phenotype was similar to a human skeletal disease called Cleido-cranial dysplasia (CCD) (Lee et al., 1997; Otto et al., 1997). It was also reported that *Cbfa-1* enhances osteoblast differentiation at an early stage but inhibited it at a later stage, as transgenic mice overexpressing *Cbfa-1* developed a high bone turnover osteopenia with numerous osteoclast invasion. The number of osteoblasts was increased but their terminal maturation and function were inhibited (Liu et al., 2001). *Cbfa-1* is also expressed in mature osteoblasts and regulates the expression of bone matrix proteins (Ducy et al., 1997). Cbfa-1 binding sites are present in the promoters of several osteoblastic genes, such as *osteocalcin*, bone sialoprotein (*Bsp*), *collagen type I* and *osteopontin*, and control their expression. Transfection of a *Cbfa1* expression vector induced the expression of *Osteocalcin* and *Bsp* in primary skin fibroblasts and fibroblastic cell lines (Ducy et al., 1997).

*Cbfa-1* affects osteoclastogenesis indirectly via osteoblasts. In *Cbfa-1* deficient mice, only some mononuclear osteoclastic cells and limited numbers of multinucleated $H^+$-ATPase positive osteoclasts with poorly developed ruffled borders were present in the perichondrium (Hoshi et al., 1999; Komori et al., 1997; Otto et al., 1997). Calvarial cells isolated form *Cbfa-1* +/+, +/- and -/- embryos were cocultured with normal spleen cells in the presence of $1,25(OH)_2D_3$ and Dexamethasone, and the number and the bone resorption activity of osteoclasts formed in coculture with mutant calvarial cells were significantly decreased in comparison with heterozygous and wild-type (Gao et al., 1998). RANKL expression was upregulated by $1,25(OH)_2D_3$ and Dexamethasone treatment in heterozygous and wild type calvarial cells, but not in homozygous mutant cells. On the other hand, OPG expression was inhibited similarly in all three genotypes (Gao et al., 1998). In contrast, osteoblastic cells overexpressing *Cbfa-1* have an increased ability to support osteoclast formation, probably by upregulating RANKL and M-CSF expression and downregulating OPG expression (Geoffroy et al., 2002). However, Thirunavukkarasu et al. reported that there are 12 putative Cbfa-1 binding elements (osteoblast-specific element 2 (OSE2) sites) pre-

sent in the OPG promoter. Overexpression of *Cbfa-1* in osteoblastic cell lines increased OPG protein levels (Thirunavukkarasu et al., 2000).

Recently, *Osterix* (*Osx*) has been identified as a transcription factor that is required for osteoblast differentiation during development and acts downstream of *Cbfa-1* (Nakashima et al., 2002). Osx is a zinc finger-containing protein, which is expressed in osteoblast progenitors. Mice lacking *Osx* developed a cartilaginous skeleton without osteoblasts, which is similar to the *Cbfa-1* knockout mice but less severe. However, the fact that *Cbfa-1* is expressed in *Osx* mutant mice, while *Osx* expression is absent in *Cbfa-1* mutant mice, suggests that *Osx* is genetically located downstream of *Cbfa-1*. In addition, fully functional osteoclasts were present in *Osx* mutant long bones.

In conclusion, *Cbfa-1* controls osteoblast differentiation which further affects osteoclastogenesis, suggesting that osteoblast are crucial for osteoclast differentiation. Not only fully differentiated osteoblasts but also osteoblast progenitors have the ability to support osteoclast formation, as osteoclastogenesis are not affected in *Osx* knockout mice.

### 1.3.2 Osteocytes and lining cells

#### 1.3.2.1 Osteocytes

Osteocytes are considered the most mature or terminally differentiated cells of the osteoblast lineage (Holtrop, 1975; Jande and Belanger, 1973). They are embedded in the bone matrix and communicate with each other and the surface lining cells via gap junctions on cytoplasmic projections within canaliculi through the mineralised matrix (Menton et al., 1984). Recently, a bone-specific cDNA named osteoblast/osteocyte factor 45 (*OF45*) was identified as an osteocyte specific marker gene, which is only expressed in cells embedded within bone matrix. This cDNA encodes a serine/glycine-rich secreted protein containing one RGD sequence motif, which may play a role in regulating bone cell activity (Petersen et al., 2000).

Osteocytes are capable of synthesising osteopontin which in turn induces osteoclasts and osteoblasts to attach to the bone matrix (Gerstenfeld, 1999). Evidence showed that osteocytes negatively regulate osteoclast function. Conditioned medium from an osteocyte cell line inhibited bone resorption of isolated osteoclasts, and this

effect was enhanced by estrogen pre-treatment and abolished by an antibody against transforming growth factor-β (TGF-β) (Heino et al., 2002).

It has been suggested that osteocytes are the mechano-sensors of bone, responding to mechanical stimuli. They signal information to the endosteal and periosteal surfaces via their cell processes in canaliculi that form a continuous network (Lanyon, 1993). Osteocytes produce nitric oxide and prostaglandins (Pitsillides et al., 1995; Rawlinson et al., 1995) upon mechanical loading. They also undergo apoptosis by damage-inducing loads, which may serve as a signal for osteoclast activation and stimulating local bone resorption (Noble et al., 2003). The responsiveness of osteocytes to mechanical loading is regulated by estrogen. Osteocytes express both α and β estrogen receptors (Vidal et al., 1999). Osteocyte apoptosis was increased by ovariectomy in rats, which was abolished by estrogen treatment, suggesting that they may play a role in estrogen deficiency-induced bone loss after menopause (Tomkinson et al., 1998).

### 1.3.2.2 Bone lining cells

Another form of terminally differentiated osteoblasts are the bone lining cells, which are flat and highly elongated with a spindle shaped nuclei (Menton et al., 1984). These cells are found on newly formed bone surfaces, but their functions are not clear. It was suggested that bone lining cells are involved in bone remodelling. During bone remodelling, targeting of osteoclast precursors to a specific location on bone depends on the "homing" signals sent from bone lining cells, which are under control of osteocytes (Parfitt et al., 1996). Chambers et al. reported that osteoblastic cells (bone lining cells) initiated bone resorption by digesting unmineralised osteoid, which protects bone mineral from osteoclastic contact (Chambers and Fuller, 1985). Bone lining cells are also thought to be able to revert to an earlier osteoblastic phenotype with parathyroid hormone (PTH) stimulation (Menton et al., 1984). After mechanical stimulation, bone lining cells changed to a cuboidal shape with rounded nuclei and abundant rough endoplasmic reticulum characteristic of osteoblasts, suggesting these cells are reactivated (Chow et al., 1998). Some studies showed that they might be reactivated to synthesise osteoid at specific times *e.g.* during fracture healing (Bland et al., 1999). It was also reported that bone lining cells cleaned up the resorption pits left by osteoclasts, which contained nondigested demineralised bone colla-

gen. This "cleaning up" is a prerequisite for the subsequent deposition of collagenous proteins by osteoblasts in the resorption pits (Everts et al., 2002).

Although osteocytes and bone lining cells are terminally differentiated osteoblasts, they play an important role in bone remodelling and osteoclastic bone resorption.

### 1.3.3 The osteoclast phenotype

Osteoclasts are the bone cells responsible for bone resorption. They are giant multinucleated cells, containing four to 20 nuclei. Osteoclasts are usually found in contact with calcified bone surfaces and within lacunae (Howship's lacunae) (Lucht, 1980). Their cytoplasm contains many vacuoles and mitochondria. The most prominent feature of active osteoclasts is the deep foldings of the plasma membrane in the area facing the bone matrix, named the ruffled border, which is surrounded by a ring of contractile proteins (sealing zone). The sealing zone serves as a barrier, preventing the leakage of secreted protons and proteases from the resorption area, and also plays a crucial role in osteoclast polarisation (Stenbeck and Horton, 2000). Formation of the sealing zone involves the rearrangement of the cytoskeleton so that actin forms a dense belt-like structure, called the "actin ring" (Lakkakorpi et al., 1989).

Osteoclasts express several unique phenotypic features that distinguish them from other multinucleated giant cells. To date, the expression of calcitonin receptors (CTR) and lacunar bone resorption are widely accepted as the two most specific features for osteoclasts. The unique ability of osteoclasts to form resorption lacunae provides a specific and reliable way of identifying these cells (Boyde et al., 1984; Chambers and Horton, 1984). Other cell types, including monocytes and macrophages, are able to dissolve both the inorganic and the organic matrix components of bone, but don't form lacunae (Teitelbaum et al., 1979).

#### 1.3.3.1 Calcitonin receptor (CTR)

Osteoclastic activity is directly and specifically inhibited by calcitonin, and the demonstration of receptors that bind calcitonin is a reliable and highly specific marker of a mammalian osteoclast (Takahashi et al., 1988a). The calcitonin receptor (CTR) belongs to a subfamily of G protein-coupled receptors. There are two different CTR isoforms expressed in murine tissues: C1a, and C1b which is primarily expressed in

brain (Pondel, 2000). CTR is expressed on committed osteoclast precursors and mature osteoclasts (Lee et al., 1995; Quinn et al., 1999). The expression of *CTR* mRNA was coincident with the development of osteoclasts in bone marrow culture and was more specific than TRAP expression (Lee et al., 1995).

### 1.3.3.2 MMP-9

The expression of matrix metalloproteinase-9 (MMP-9) is the earliest marker for cells in the osteoclast lineage (Roodman, 1999). MMP-9 (92kDa type IV collagenase /gelatinase B) cleaves native collagens of type IV, V and XI, and elastin. It has a role in the removal of denatured collagen fragments following the action of cysteine proteinases as an early step in bone resorption (Reponen et al., 1994; Wucherpfennig et al., 1994). During embryonic development, *MMP-9* is highly expressed by trophoblast cells at the implantation site and by osteoclasts (Alexander et al., 1996; Reponen et al., 1994). MMP-9 plays an important role in the migration of preosteoclasts from the perichondrium into the cartilaginous bone model, to transform it into the bone marrow cavity during endochondral ossification (Blavier and Delaisse, 1995). Mice deficient in *MMP-9* are viable but exhibit a delay in osteoclast recruitment, resulting in delayed endochondral ossification and an excessively wide zone of hypertrophic cartilage, with no effects on intramembranous bone formation (Engsig et al., 2000). The differentiation of hypertrophic chondrocytes was normal, but apoptosis, vascularisation and ossification were delayed. *In vitro* angiogenesis assays showed that *MMP-9* mutant cartilage induced a delayed angiogenic response, suggesting the release of an angiogenic activator, identified as VEGF, is delayed in the absence of MMP-9 (Vu et al., 1998). Transplantation of wild-type bone marrow cells rescued the defects of vascularisation and ossification, suggesting the defect was intrinsic to haematopoietic cells.

### 1.3.3.3 TRAP

Osteoclasts contain high levels of acid hydrolases. One of them is Tartrate-resistant acid phosphatase (TRAP) (purple acid phosphatase, type 5 acid phosphatase, Acp5). TRAP is expressed in a variety of tissues, but bone expresses the highest level among normal tissues. In bone, TRAP is highly expressed in osteoclasts and is present in lysosomes, Golgi, extracellular channels of the ruffled border, and the

space between the cells and bone (Hammarstrom et al., 1971; Minkin, 1982). TRAP is considered as a reliable marker for osteoclasts.

The function of TRAP in osteoclasts is not clear. It has been reported that TRAP can dephosphorylate osteopontin and bone sialoprotein, which have been implicated in cell attachment (Ek-Rylander et al., 1994). *TRAP*-deficient mice have a mild osteopetrotic phenotype due to an intrinsic defect of osteoclastic remodelling activity. Osteoclasts were present in *Acp5-/-* long bones, but their resorptive function was impaired (Hayman et al., 1996), suggesting that TRAP may play an important role in osteoclastic bone resorption.

### 1.3.3.4 Cathepsin K

Cathepsin K is a cysteine protease of the papain family of proteases that is abundantly and selectively expressed within osteoclasts, while other cystein proteinases (cathepsin B, H, L or S) are absent or expressed at very low levels (Drake et al., 1996; Soderstrom et al., 1999). It is a 39kDa protein synthesised as a pre-proenzyme that can be converted autocatalytically to the mature enzyme form (McQueney et al., 1997). *Cathepsin K* expression during embryogenesis is restricted to osteoclasts and preosteoclasts at sites of active cartilage and bone remodelling (Dodds et al., 1998). *In situ* hybridisation showed that *cathepsin K* is highly and specifically expressed in human and rabbit osteoclasts (Drake et al., 1996; Tezuka et al., 1994). It is secreted by activated osteoclasts into the resorption lacunae on bone surface (Yamaza et al., 1998).

Cathepsin K functions as a matrix-degrading proteinase in bone resorption. It has both gelatinase and collagenase activity and can effectively degrade types I and II collagen, osteopontin and osteonectin at low pH (Garnero et al., 1998). *Cathepsin K* knockout mice developed pronounced osteopetrosis and extramedullary haematopoiesis. Histological analysis showed that the number of osteoclasts was not decreased, but that these osteoclasts failed to resorb and endocytose the bone matrix (Gowen et al., 1999). The resorptive surface of bone in the mutant mice was poorly defined with a broad demineralised matrix fringe containing undigested fine collagen fibrils (Saftig et al., 1998), suggesting *cathepsin K* deficient osteoclasts are capable of demineralising the extracellular matrix but are unable to adequately remove the demineralised bone (Gowen et al., 1999). *Cathepsin K* deficient osteoclasts had irregular ruffled borders,

detached sealing zone and large irregular vacuoles in the cytoplasm, resulting in a severely impaired function. A deletion of *cathepsin K* has been related to a human disease called pycnodysostosis with short stature and osteosclerosis (Gelb et al., 1996).

Mice overexpressing the *Ctsk* gene, which codes for cathepsin K, developed high turnover osteoporosis with increased number of osteoblasts. Furthermore, immunohistochemistry showed that cathepsin K expression was confined to osteoclasts. These results suggested that bone resorption and formation are tightly coupled during bone remodelling (Kiviranta et al., 2001).

### 1.3.3.5 Carbonic anhydrase II

Osteoclasts express high levels of carbonic anhydrase II. Carbonic anhydrase II (CAII) is an enzyme catalysing the association of $CO_2$ and $H_2O$ to form $H_2CO_3$, which dissociates to $H^+$ and $HCO3^-$. Osteoclasts actively resorbing bone express higher levels of *CAII* than resting osteoclasts (Asotra et al., 1994). 1, 25$(OH)_2D_3$ and PTH stimulate *CAII* expression in bone marrow cultures, which could be due to the stimulatory effect on osteoclast differentiation (Biskobing et al., 1997). CAII plays an important role in osteoclastic bone resorption. Antisense constructs against *CAII* block osteoclastic bone resorption both in isolated osteoclasts and in bone organ cultures (Laitala and Vaananen, 1994; Laitala-Leinonen et al., 1999). Congenital absence of *CAII* in children results in osteopetrosis, renal tubular acidosis and cerebral calcification (Sly et al., 1983). Osteoclasts in these patients fail to develop a ruffled border and are unable of resorb bone (Marks, 1989). Bone marrow transplantation cured the osteoclast defect and osteopetrosis, but not the renal lesions (McMahon et al., 2001), suggesting that the skeletal defects are intrinsic to the haematopoietic lineage. It was also reported that CAII plays an important role in osteoclast differentiation through pH regulation. CAII specific inhibitors suppressed 1,25$(OH)_2D_3$, induced osteoclast formation in a dose-dependent manner (Lehenkari et al., 1998).

### 1.3.3.6 $H^+$-ATPase

The central role of activated osteoclasts is acid secretion via the highly expressed vacuolar (V-type) electrogenic $H^+$-ATPase. The V-ATPase-deficient *atp6i* mice (Li et al., 1999b), which carry the same mutation as the spontaneous *oc/oc* mouse (Scimeca et al., 2000), also have dysfunctional osteoclasts, which are unable to

acidify the resorption lacuna. Disruption of a late endosomal/lysomal $Cl^-$ channel, CIC-7 $Cl^-$ channel, which provide the chloride conductance required for efficient proton pumping by the $H^+$-ATPase, results in a phenotype similar to the *atp6i* mice (Kornak et al., 2001). Osteoclasts are present in both mutant long bones, but they fail to resorb bone due to the deficiency in acidifying the extracellular resorption lacuna.

### 1.3.3.7 Vitronectin receptor

Integrins are a superfamily of cell surface receptors composed of non-covalent heterodimers containing α and β subunits. There are at least 15 different α chains and 8 different β chains have been identified. The extracellular domain of many integrins interacts with the RGD (Arg-Gly-Asp) tripeptide found in many extracellular proteins, such as vitronectin, fibronectin, osteopontin and bone sialoprotein. Integrins mediate cell-matrix and cell-cell interactions (Hynes, 1992). Mammalian osteoclasts express high levels of $\alpha_v\beta_3$, the vitronectin receptor; also $\alpha_2\beta_1$, a collagen/laminin receptor and $\alpha_v\beta_1$, another vitronectin receptor. At the ultrastructural level, $\alpha_v\beta_3$ is localised to the ruffled border, the basolateral membrane (Lakkakorpi et al., 1993) and the clear zone (Reinholt et al., 1999).

Integrins regulate osteoclast activity by mediating osteoclast adhesion and regulating the cytoskeletal organisation required for both cell migration and formation of the sealing zone (Nakamura et al., 1999). The vitronectin receptor, $\alpha_v\beta_3$, mediates the attachment of osteoclasts to bone matrix by binding to osteopontin (Ross et al., 1993). RGD-containing peptides, which can inhibit the function of $\alpha_v\beta_3$, inhibit osteoclast resorption *in vitro* by disrupting the ruffled border and clear zone (Nakamura et al., 1996; Sato et al., 1990). Monoclonal anti-vitronectin receptor $\alpha_v\beta_3$ complex antibody induced significant osteoclast retraction in chick osteoclasts and inhibited their resorption (Horton et al., 1991). Inhibition of $\alpha_v\beta_3$ integrins also inhibits osteoclastic bone resorption *in vivo*. RGD-containing peptides and a monoclonal antibody to $\beta_3$ subunits blocked the PTH-induced increase in serum calcium in thyroparathyroidectomised (TPTX) animals (Crippes et al., 1996; Fisher et al., 1993). Synthetic RGD mimetics prevent the rapid bone loss after estrogen withdrawal, suggesting that $\alpha_v\beta_3$ blockade may prevent postmenopausal osteoporosis (Engleman et al., 1997). Mice lacking $\beta_3$ developed osteosclerosis and hypocalcaemia with age. The number of osteoclasts was increased in the mutant mice, but they were dysfunctional. Osteo-

clasts isolated from these mice failed to spread, lacked the actin rings and exhibited reduced bone resorption activity *in vitro* (McHugh et al., 2000).

Integerin $\alpha_v\beta_3$ also plays a role in osteoclast differentiation. RGD-containing peptides inhibited multinucleated osteoclast formation by preventing M-CSF-induced migration and fusion of preosteoclasts in a co-culture system (Boissy et al., 1998; Nakamura et al., 1998). On the other hand, Miyamoto et al. reported that RGD-containing peptides and antibodies against $\alpha_v\beta_3$ inhibited multinucleation of TRAP positive cells in a dose-dependent manner by downregulating cell proliferation, rather than affecting the initial attachment (Miyamoto et al., 2000). Under normal *in vitro* culture conditions, bone marrow macrophages from $\beta_3$ knock out mice formed much less multinucleated osteoclasts than the wild-type, and these cells were dysfunctional as well (McHugh et al., 2000). High dose M-CSF completely rescued the $\beta_3$-/- osteoclastogenesis defect, but not the resorption function, by activating the ERK (extracellular signal-regulated kinases)/c-Fos signalling pathway (Faccio et al., 2003).

Activated $\alpha_v\beta_3$ exerts its biological effects through Pyk2/Src/Cbl (Sanjay et al., 2001), $p130^{cas}$ (cas, Crk associated substrate) (Lakkakorpi et al., 1999), PI3-K (phosphatidyl inositol 3-kinase) (Chellaiah et al., 1998) and Rho (Chellaiah et al., 2000) signalling pathways.

#### 1.3.3.8 RANK

RANK (receptor activator of NF-κB), also named osteoclast differentiation and activation receptor (ODAR) is the receptor for RANKL. It was originally identified in a dendritic cell cDNA library (Anderson et al., 1997). RANK is a type I transmembrane protein. It is present on osteoclasts and their precursors (Myers et al., 1999; Nakagawa et al., 1998), and mediates the function of RANKL in osteoclast differentiation and activation (Hsu et al., 1999). The expression of RANK is upregulated by M-CSF (Arai et al., 1999; Lacey et al., 1998). Antibodies against the extracellular domain of RANK stimulated osteoclast formation *in vitro* (Hsu et al., 1999; Nakagawa et al., 1998).

Deletion of *RANK* leads to severe osteopetrosis (Li et al., 2000). The mutant mice lack osteoclasts and have a profound defect in bone resorption and remodelling and in the development of the cartilaginous growth plates of endochondral bone.

They also exhibit a marked deficiency of B cells in the spleen and have a lymph node deficiency, whereas macrophages and dendritic cells are not affected (Dougall et al., 1999). Spleen cells from *RANK*-/- mice could not form osteoclasts *in vitro* in the presence of RANKL and M-CSF, but this could be rescued when these cells were infected with a *RANK*-expressing retrovirus. Bone marrow transplantation from *rag1*-/- mice, which have normal osteoclast precursors, cured the osteopetrosis, suggesting that the defect was restricted to the osteoclast lineage. Injection of PTHrP, $1,25(OH)_2D_3$ and IL-1 into *RANK* mutant mice didn't induce osteoclastic bone resorption, suggesting that the proresorptive activity of these factors is mediated by the RANK signalling pathway. Surprisingly, injection of TNF-α induced rare TRAP and cathepsin K positive osteoclast formation on the bone surface, suggesting that TNF-α may act through an alternative compensatory pathway to stimulate osteoclast differentiation in the absence of RANK (Li et al., 2000). Transgenic mice expressing a soluble form of RANK, which binds to RANKL with high affinity, developed osteopetrosis due to a reduction in osteoclastogenesis, similar to OPG transgenic mice (Hsu et al., 1999) (see also below).

In conclusion, although some of the markers shown above are not complete osteoclast specific, when used in combination, they specifically localise osteoclasts both *in vivo* and *in vitro*, and are of great benefit to osteoclast studies.

### 1.3.4 Osteoclastic bone resorption

Osteoclastic bone resorption is a multistep process involving recognition, attachment to bone surface, establishment of cell polarity, migration, and subsequent degradation of bone matrix components. Bone resorption depends on the ability of the osteoclast to generate an acid extracellular compartment between itself and the bone surface. Acidic pH is essential for solubilisation of the alkaline salts of bone mineral as well as digestion of the organic bone matrix by acid lysosomal enzymes secreted by osteoclasts. The combination of these two processes is responsible for bone resorption. Carbonic anhydrase II catalyses the formation of carbonic acid ($H_2CO_3$) from water and carbon dioxide. Carbonic acid spontaneously dissociates to bicarbonate $HCO_3^-$ and $H^+$. The protons are subsequently transported through the cell membrane to the bone surface by $H^+$-ATPase, located in the ruffled border. While protons are extruded into the resorption lacuna, chloride ions are translocated to the

bone site through a $Cl^-$ channel, resulting in a net transport of hydrochloric acid. Hydroxyapatite crystals are dissolved by the acidic microenvironment under osteoclasts. The bicarbonate remaining in the cytoplasm is eliminated from the cell via a $HCO3^-$/ $Cl^-$ exchanger, replacing the $Cl^-$ secreted into the resorption lacuna. Subsequently, the organic components exposed after dissolution of the mineral are digested via lysosomal enzymes and collagenase, which are excreted into the lacuna by osteoclasts. Cysteine proteinases are activated first at low pH and digest part of the bone matrix. After the pH has increased somewhat, MMPs digest the rest of the matrix (Everts et al., 1998). Additionally, actively resorbing osteoclasts release oxygen-derived radicals, especially superoxide, which probably contribute to the degradation of the bone matrix (Felix et al., 1996; Rousselle and Heymann, 2002). Mouse bone explants experiment showed that osteoclastic resorption of calvarial bone depends on activity of both cysteine proteinases and MMPs, while long bone resorption depends only on cysteine proteinases (Everts et al., 1999). After extracellular digestion, the degraded products are removed by endocytosis and vesicular transcytosis through osteoclasts (Palokangas et al., 1997; Zhao et al., 2002) (Fig.1.4)(Adapted from Stenbeck, 2002).

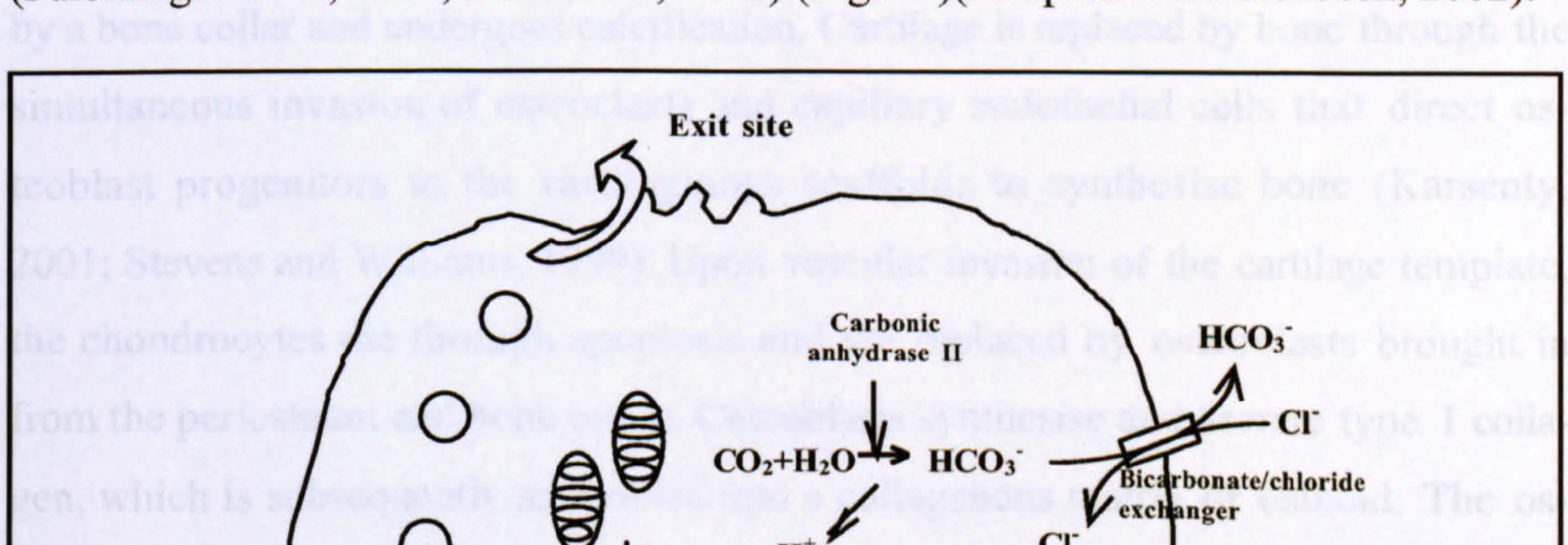



**Fig1. 4 Mechanism of osteoclastic bone resorption**

## 1.4 Bone development and remodelling

There are two types of processes involved in bone formation: intramembranous ossification (flat bones) and endochondral ossification (long bones).

### 1.4.1 Intramembranous ossification

In intramembranous ossification, a group of mesenchymal cells within a highly vascularised area of the embryonic connective tissue proliferates and differentiates directly into preosteoblasts and then into osteoblasts. These cells synthesise a bone matrix with irregular collagen fibre bundles, numerous osteocytes and irregularly distributed ossification patches. This type of bone is called woven bone. Later, this woven bone is progressively replaced by mature lamellar bone.

### 1.4.2 Endochondral ossification

In endochondral ossification, mesenchymal cells proliferate and differentiate along chondrocytic lineage. The chondrocytes secrete cartilaginous matrix and form a cartilage template which prefigures the future bone. The cartilage is then surrounded by a bone collar and undergoes calcification. Cartilage is replaced by bone through the simultaneous invasion of osteoclasts and capillary endothelial cells that direct osteoblast progenitors to the cartilaginous scaffolds to synthesise bone (Karsenty, 2001; Stevens and Williams, 1999). Upon vascular invasion of the cartilage template, the chondrocytes die through apoptosis and are replaced by osteoblasts brought in from the periosteum and bone collar. Osteoblasts synthesise and secrete type I collagen, which is subsequently assembled into a collagenous matrix or osteoid. The osteoid layer then calcified by the process of mineralisation (Ducy et al., 2000).

### 1.4.3 Bone remodelling

Throughout the life, the vertebrate skeleton is under constant bone remodelling, which is crucial for maintaining a well-adapted skeleton. Bone remodelling is a very complex process of tightly coordinated action by the bone resorbing osteoclasts and the bone forming osteoblasts. The two processes are usually tightly regulated by a complex interplay of cell-cell and cell-matrix interactions involving the production of enzymes and activation of inhibitors and regulatory molecules such as cytokines and growth factors. Dysfunction of and imbalance between osteoblasts and osteoclasts lead to bone metabolic diseases, such as osteoporosis in which bone resorption ex-

ceeds formation, or in contrast, osteopetrosis in which bone formation exceeds resorption.

In cortical bone, bone remodelling is carried out by a structure called basic multicellular unit (BMU) (Parfitt et al., 1996). A typical BMU comprises osteoclasts in the front, osteoblasts in the rear, and a central vascular capillary, a nerve supply and connective tissues in the middle. All remodelling begins on a quiescent surface, which is covered by bone lining cells. Bone lining cells produce collagenase and digest the layer of unmineralised matrix so that the bone surface is exposed to osteoclasts (Chambers et al., 1985a). Inactivated osteoclast precursors migrate to the site that is to be resorbed in response to some unknown signals. Mesenchymal cells synthesise cytokines that induce and modulate growth and differentiation of osteoclast precursors to mature osteoclasts (Burger et al., 1984). After adhesion to the mineralised bone matrix, activated osteoclasts form a tight sealing zone that enclose the resorption lacunae and create an isolated microenvironment, where the low pH and high concentration of enzymes are maintained. The ruffled border develops beneath the osteoclast surrounded by the sealing zone. Osteoclasts resorb bone by producing and releasing hydrogen ions, lactate and proteolytic enzymes to the subcellular space. As the BMU progresses through the surface of bone, the transition from resorption to formation occurs (Parfitt, 1994). Osteoclastic bone resorption is terminated by osteoclast apoptosis (Parfitt et al., 1996), the surface of the resorption cavity becomes smoother and covered by a thin layer of cement substance and mononuclear cells. Finally, mesenchymal precursors are recruited to the resorption site and differentiate into osteoblasts. Osteoblasts lay down a protein matrix filling in the lacunae, called osteoid, which then becomes calcified. After bone formation, some osteoblasts become flat and line the quiescent bone surfaces, that is, they become bone lining cells (Fig.1.5) (Adapted from Christenson, 1997).

In cancellous bone, a bone remodelling compartment (BRC) is associated with bone remodelling (Hauge et al., 2001). The difference between BMU and BRC is the maintenance of the bone lining cell barrier throughout bone remodelling. After the digestion of unmineralised matrix, bone lining cells become the marrow lining cells, allowing bone resorption and bone formation to proceed under a common roof of lining cells. At the end of bone formation, new lining cells derived from osteoblasts

replace the marrow lining cells and close the BRC. The two layers of lining cells eventually become a single layer.

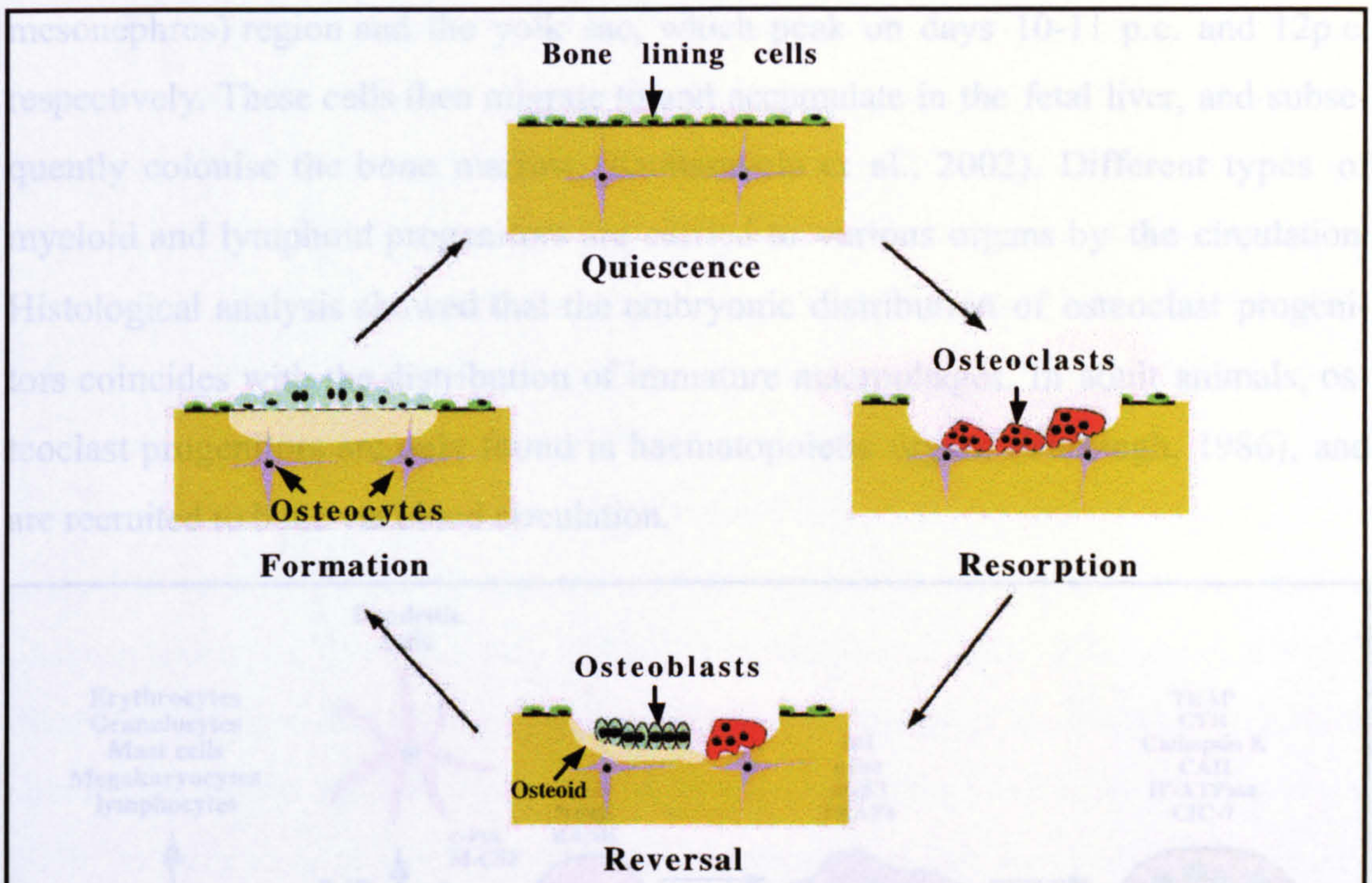



**Fig 1.5 Bone remodelling cycle**

The mechanism of coupling between bone resorption and bone formation is not clear. It was suggested that growth factors such as TGF-β are stored in bone matrix and would be released during bone resorption (Bonewald and Mundy, 1990). The capillary in the middle of BMU may also play a role in coupling. Vascular pericytes could serve as a source of osteoblasts. Cultured pericytes expressed many features of the osteoblast phenotype and have been reported to have the capacity to form nodules (Brighton et al., 1992).

## 1.5 Modulation of osteoclast differentiation

Osteoblasts and osteoclasts are equally important for maintaining healthy skeleton. Since this thesis focuses mainly on osteoclasts, this next section will review the many advances that have been made in the last decades in osteoclast biology.

### 1.5.1 Origin of osteoclasts

It is widely accepted that osteoclasts are haematopoietic in origin and are derived from cells in the monocyte/macrophage lineage (Fig.1.6)(Adapted from

Karsenty and Wagner, 2002). During early embryogenesis, haematopoietic stem cells are initially generated and expanded in the intraembryonic AGM (aorta-gonads-mesonephros) region and the yolk sac, which peak on days 10-11 p.c. and 12p.c. respectively. These cells then migrate to and accumulate in the fetal liver, and subsequently colonise the bone marrow (Kumaravelu et al., 2002). Different types of myeloid and lymphoid progenitors are carried to various organs by the circulation. Histological analysis showed that the embryonic distribution of osteoclast progenitors coincides with the distribution of immature macrophages. In adult animals, osteoclast progenitors are only found in haematopoietic organs (Thesingh, 1986), and are recruited to bone via blood circulation.

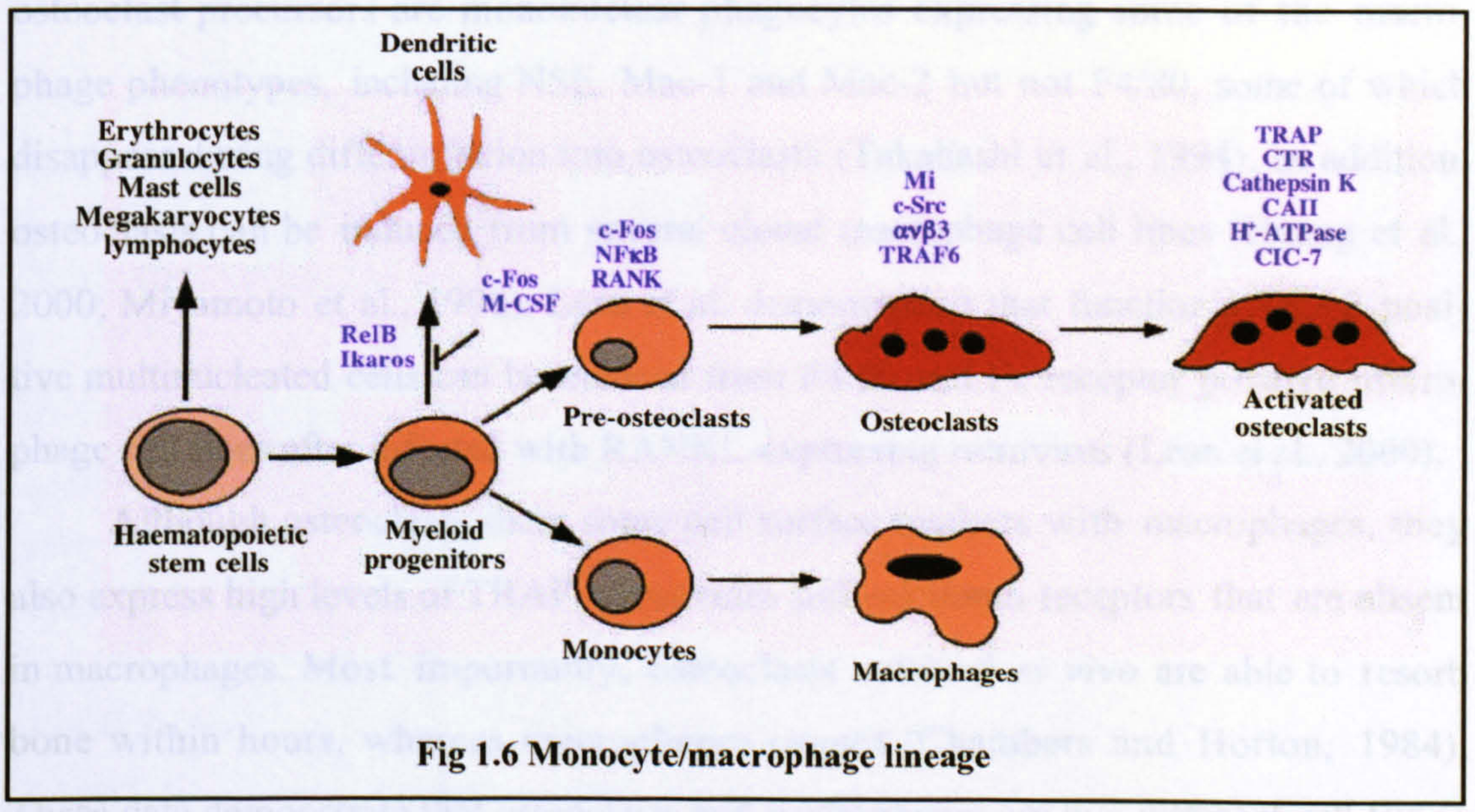



**Fig 1.6 Monocyte/macrophage lineage**

Several lines of evidence have suggested that the osteoclast derives from cells in the monocyte/macrophage lineage. The multipotential precursors divert to different pathways by the regulation of growth factors secreted by osteoblastic/stromal cells in the bone marrow (Shinar et al., 1990). Osteoclasts and macrophage polykaryons are both formed by fusion of mononuclear precursors (Zambonin Zallone et al., 1984), and there are several ultrastructural similarities between them. In addition, both osteoclasts and macrophages show trypsin-resistant adherence to a glass or plastic substrate (Chambers, 1979). Furthermore, osteoclasts share some immunophenotypic characteristics with monocytes and macrophages. Tsurukai et al. reported that osteoclast precursors are positive for Mac-1, Mac-2 and Gr-1, but negative for F4/80, B220 and CD3e (Tsurukai et al., 1998). Niida et al. also showed that both

preosteoclasts and osteoclasts highly expressed Mac-2, but not MOMA-2, Mac-1, F4/80 or BM8 (Niida et al., 1994).

*In vivo* experiments showed that the mononuclear cells that initially attached to the bone surface contained non-specific esterase, a macrophage marker. These cells expressed TRAP after they differentiated, and eventually lost their non-specific esterase activity and formed multinucleated osteoclasts (Baron et al., 1986). Many *in vitro* studies showed that osteoclasts can be generated not only from immature non-adherent M-CSF-dependent bone marrow cells (Quinn et al., 1998c), but also from monocytes, resident peritoneal or alveolar macrophages and tumour-associated macrophages (Fuller and Chambers, 1998). Takahashi et al. reported that postmitotic osteoclast precursors are mononuclear phagocytes expressing some of the macrophage phenotypes, including NSE, Mac-1 and Mac-2 but not F4/80, some of which disappear during differentiation into osteoclasts (Takahashi et al., 1994). In addition, osteoclasts can be induced from several clonal macrophage cell lines (Huang et al., 2000; Miyamoto et al., 1998). Lean et al. demonstrated that functional TRAP positive multinucleated cells can be induced from F4/80 and Fc receptor positive macrophage cell lines after infected with RANKL-expressing retrovirus (Lean et al., 2000).

Although osteoclasts share some cell surface markers with macrophages, they also express high levels of TRAP, vitronectin and calcitonin receptors that are absent in macrophages. Most importantly, osteoclasts cultured *ex vivo* are able to resorb bone within hours, whereas macrophages cannot (Chambers and Horton, 1984). These data demonstrate that osteoclasts and macrophages are two different cell types in spite of all these similarities.

Even though it is well established that osteoclasts and macrophages are derived from the same progenitors, how osteoclast precursors diverge from the macrophage lineage is not fully understood. It has been reported that osteoclasts derive from immature c-Kit$^{+}$ haematopoietic cells and pass through a *c-fms*-dependent stage at some point during differentiation (Hayashi et al., 1997; Sudo et al., 1995). Early osteoclast precursors are c-Kit$^{+}$Mac-1$^{dull}$c-Fms$^{+}$ cells. These cells express RANK after M-CSF stimulation, and differentiate into osteoclasts in the presence of RANKL and M-CSF, but differentiate into macrophages with M-CSF only. RANK$^{-}$ cells differentiate into osteoclasts more efficiently than RANK$^{+}$, as their proliferative activity is higher (Arai et al., 1999). Takeshita et al. demonstrated that early osteoclast progenitors in

bone marrow are mainly populated from Mac-1$^-$F4/80$^{dull}$ cells which differentiate into M-CSF-dependent bone marrow macrophages (the osteoclast progenitors) expressing Mac-1$^+$F4/80$^{intermediate}$ in response to M-CSF stimulation (Takeshita et al., 2000).

Taken together, osteoclasts and macrophages are derived from the same haematopoietic progenitors, but differentiate into specific cell lineages in response to different stimuli.

### 1.5.2 Factors that affect osteoclast formation and function

Osteoclast differentiation and activation are tightly controlled *in vivo* by cellular and hormonal factors. Table1.1 summarises the effects of the systemic and local factors on osteoclast formation and function.

**Table 1.1 Factors regulating osteoclast formation and function**

| Factors | Formation | Resorption | References |
|---|---|---|---|
| **Systemic hormones** | | | |
| PTH/PTHrP | increase | increase | (Chambers et al., 1985b; Ihara et al., 2001) |
| Calcitriol | increase/ decrease | increase | (Kurihara and Roodman, 1990; Sakai et al., 2001; Shibata et al., 2002) |
| calcitonin | decrease | decrease | (Chambers and Magnus, 1982; Gorn et al., 1995) |
| estrogen | decrease | decrease | (Manolagas, 2000) |
| $PGE_2$- mouse | increase | increase | (Takahashi et al., 1988b) |
| human | decrease | decrease | (Chenu et al., 1990) |
| **Local factors** | | | |
| RANKL | increase | increase | (Fuller et al., 1998; Lacey et al., 1998) |
| M-CSF | decrease | increase | (Fuller et al., 1993; Udagawa et al., 1999) |
| TNF-α | increase | increase | (Fuller et al., 2002; Lam et al., 2000) |
| IL-1 | increase | increase | (Azuma et al., 2000; Fuller et al., 2002) |
| IL-6 | increase | increase | (Devlin et al., 1998; Poli et al., 1994) |
| IL-11 | increase | increase | (Ahlen et al., 2002; Quinn et al., 2000) |
| TGF-β-direct | increase | increase | (Fuller et al., 2000b; Koseki et al., 2002) |
| indirect | decrease | decrease | (Chenu et al., 1988; Hughes et al., 1996; Takai et al., 1998) |
| OPG | decrease | decrease | (Fuller et al., 1998; Lacey et al., 1998) |
| IFN-γ | decrease | decrease | (Fox and Chambers, 2000; Gowen and Mundy, 1986) |
| IL-3 | decrease | decrease | (Shinar et al., 1990) |
| IL-4 | decrease | decrease | (Nakano et al., 1994; Riancho et al., 1993) |
| IL-18 | decrease | decrease | (Horwood et al., 1998b) |
| Nitric oxide | - | decrease | (Brandi et al., 1995) |

Among these factors, M-CSF and RANKL are indispensable for osteoclastogenesis (Fig.1.7)(Adapted from Kong et al., 2000). In the bone microenvironment, the relative ratio of RANKL: OPG is more important than the concentration of RANKL *per se* in osteoclast differentiation (Hofbauer et al., 2000).

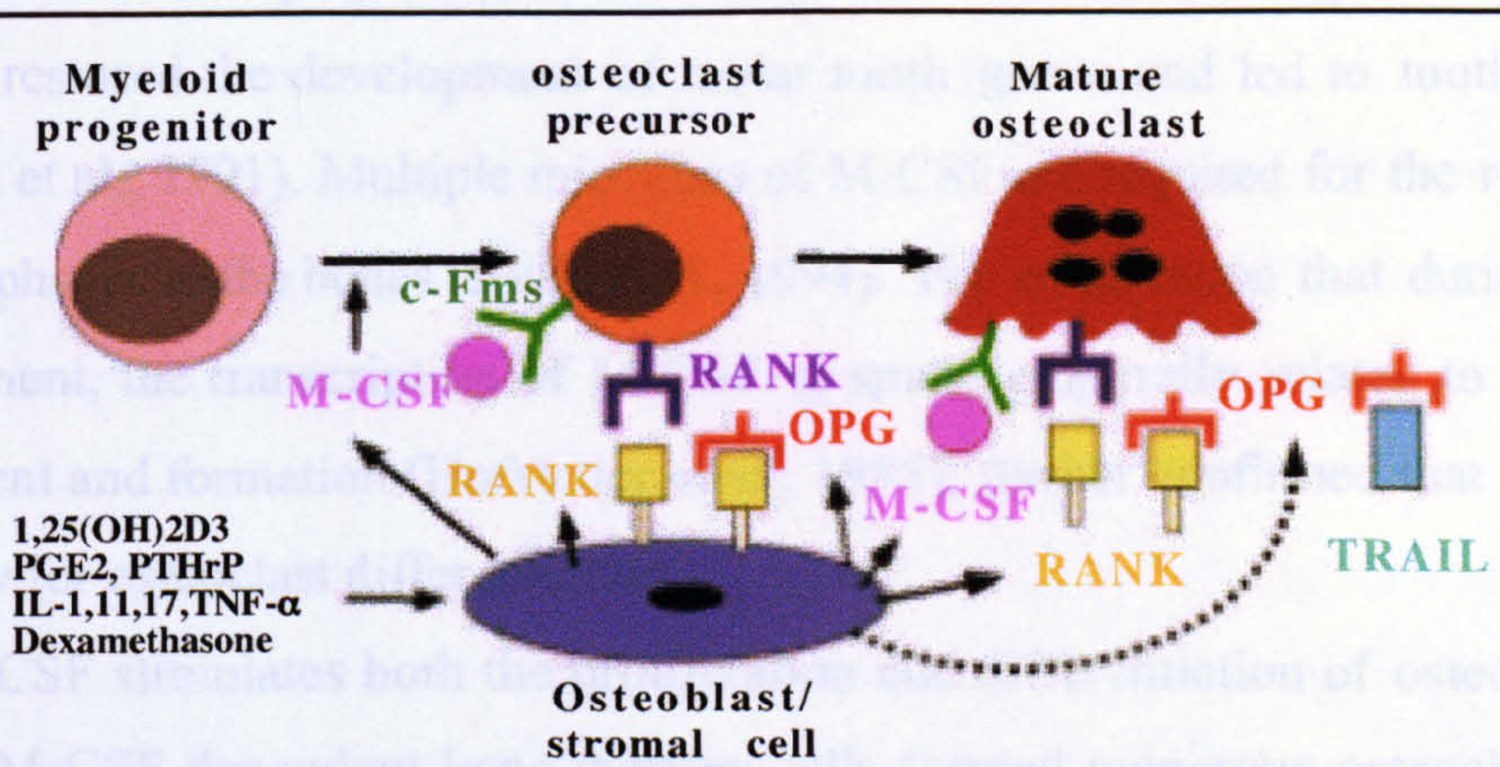



**Fig1.7 Regulation of osteoclast differentiation and function**

### 1.5.2.1 Macrophage Colony Stimulating Factor (M-CSF)

Macrophage Colony Stimulating Factor (M-CSF), also called CSF-1, is one of the Colony Stimulating Factors (CSFs). CSFs are haematopoietic growth factors that induce the clonal growth of haematopoietic progenitors both *in vitro* and *in vivo*. M-CSF is synthesised as a secreted or membrane-bound protein by macrophages and stromal cells, endothelial cells and T lymphocytes in the bone marrow microenvironment. The M-CSF receptor, encoded by the proto-oncogene *c-fms*, is a transmembrane glycoprotein with ligand-induced tyrosine kinase activity (Sherr et al., 1985). The fact that *c-fms* is expressed by osteoclast precursors and mature osteoclasts suggests that M-CSF may affect osteoclast formation and function directly (Hofstetter et al., 1992; Weir et al., 1993).

The essential role of M-CSF in osteoclastogenesis is unequivocally demonstrated in mice lacking M-CSF. Mice deficient in *M-CSF*, named *op/op* mice which have a point mutation in the coding region of *M-CSF* gene, develop osteopetrosis due to a deficiency of osteoclast (Begg et al., 1993; Yoshida et al., 1990). These mice also have reduced numbers of monocytes and macrophages. When osteoblasts from *op/op* mice cocultured with wild type spleen cells, no osteoclasts were induced without

exogenous M-CSF. Osteoclasts were generated in the coculture of wild-type osteoblasts and spleen cells from *op/op* mice (Takahashi et al., 1991a). These data suggest that the haematopoietic stem cells in *op/op* mice were not affected, and the osteopetrosis was due to the defect of M-CSF production in osteoblasts. A single injection of M-CSF into *op/op* mice cured the skeletal sclerosis (Sundquist et al., 1995), and also restored the development of molar tooth germs and led to tooth eruption (Kodama et al., 1991). Multiple injections of M-CSF are required for the recruitment of macrophages in the bones (Niida et al., 1994). The observation that during skeletal development, the transcription of M-CSF is spatiotemporally related to osteoclast recruitment and formation (Hofstetter et al., 1995), further confirmed that M-CSF is necessary for osteoclast differentiation.

M-CSF stimulates both the proliferation and differentiation of osteoclast precursors. M-CSF-dependent bone marrow cells formed numerous osteoclasts when cocultured with osteoblasts in the presence of $1,25(OH)_2D_3$ (Takahashi et al., 1991b). Osteoclastogenesis was significantly reduced by an anti-c-Fms antibody at later stages of differentiation in embryonic stem (ES) cell and in a stromal cell coculture system (Yamane et al., 1997). In a human bone marrow culture system, addition of M-CSF enhanced osteoclast formation and bone resorption in a dose- and time-dependent manner in the presence of $1,25(OH)_2D_3$ (Sarma and Flanagan, 1996).

In addition to its effects on osteoclast precursors, M-CSF also affects mature osteoclasts. M-CSF stimulated bone resorption of primary osteoclasts isolated from rabbit by increasing the average size of osteoclasts (Lees and Heersche, 1999). However, Corboz et al. reported that M-CSF stimulated bone resorption by increasing the number of osteoclasts in a bone explant experiment (Corboz et al., 1992). Recently, it was identified that M-CSF also plays a role in maintaining the survival and chemotactic behaviour of mature osteoclasts. M-CSF prevented osteoclast apoptosis, enhanced motility, and inhibited bone resorption (Fuller et al., 1993; Hattersley et al., 1988; Udagawa et al., 1999).

It was reported that osteopetrosis in *op/op* mice improved with age, suggesting that M-CSF is required for early osteoclast development, but other cytokines can compensate the effects at later stage (Begg et al., 1993; Nilsson et al., 1995). Recent experiments showed that vascular endothelial growth factor (VEGF) is one of the candidates. One single injection of VEGF induced osteoclasts recruitment in *op/op*

mice similar to M-CSF (Niida et al., 1999). Osteoclasts predominantly express VEGF receptor 1 (VEGFR-1), and VEGF increased bone resorption of primary osteoclasts in a dose-dependent manner *in vitro*, partially by prolonging their survival (Nakagawa et al., 2000). In addition, VEGF also plays an important role in osteoclast recruitment during embryonic bone development, as soluble VEGF receptor inhibited osteoclast invasion into hypertrophic cartilage (Engsig et al., 2000).

SHP-1, Src-homology domain-2 phosphatase 1 (SHP-1) (also known as haematopoietic cell phosphatase, PTP-1C, src homology PTP-1, or PTP nonreceptor type 6), is a cytoplasmic protein-tyrosine phosphatase (PTP). It has been reported that SHIP-1 is a negative regulator of M-CSF signalling in osteoclast. The motheaten ($me^v$) mutation resulted in a partial loss of SHP-1 catalytic activity and led to osteoporosis in mice. The double homozygous $me^v/me^v/op/op$ mice displayed less severe osteopetrosis than *op/op* mice. TRAP positive mononuclear cells were present on the endosteal surface of double mutant long bones, suggesting that M-CSF-independent mechanisms for osteoclastogenesis might be present in $me^v/me^v/op/op$ mice to compensate for the absence of M-CSF (Umeda et al., 1999).

#### 1.5.2.2 Receptor Activator of NF-κB Ligand (RANKL)

Receptor activator of NF-κB ligand (RANKL), also named ODF (osteoclast differentiating factor) was found by screening a cDNA expression library of ST2 cells treated with $1,25(OH)_2D_3$ and Dexamethasone using OPG as a probe (Lacey et al., 1998; Yasuda et al., 1998b). It is identical to TRANCE (TNF-related activation-induced cytokine), and osteoprotegrin (OPG) ligand, which were cloned as factors regulating T-cell and dendritic-cell functions (Anderson et al., 1997; Wong et al., 1997a). RANKL is a type II transmembrane protein, and belongs to the TNF ligand family. It was reported that RANKL is present in both membrane-bound and soluble forms (Lacey et al., 1998), and it can be released from the cell surface by a metalloproteinase (Lum et al., 1999). *RANKL* is expressed by a number of different cell types. The highest mRNA levels are in trabecular bone and lymphoid tissues (Wong et al., 1997a; Wong et al., 1997b; Yasuda et al., 1998b). In bone tissues, during mouse embryogenesis, RANKL mRNA is expressed in prehypertrophic and hypertrophic chondrocytes. In newborn and adult mouse, high levels of *RANKL* mRNA are expressed in mesenchymal cells of the periosteum and mature osteoblasts. Unexpect-

edly, osteoclasts within resorption lacunae show strong *RANKL* expression (Kartsogiannis et al., 1999). Osteoblast/stromal cell lines that could support osteoclast formation in cocultures also express *RANKL* (Lacey et al., 1998).

RANKL mediates the cell-to-cell signals responsible for osteoclastogenesis (Yasuda et al., 1998a). RANKL acts directly on osteoclast precursors and stimulate osteoclastogenesis. In the presence of M-CSF, RANKL is sufficient to support osteoclast differentiation *in vitro* from human peripheral blood mononuclear cells, mouse bone marrow and spleen cells and macrophage cells lines in the absence of osteoblast/stromal cells (Lacey et al., 1998; Matsuzaki et al., 1998; Quinn et al., 1998a; Yasuda et al., 1998b). RANKL also plays an important role in osteoclast activation and survival. RANKL stimulated bone resorption of mouse and rat mature osteoclasts *in vitro* (Burgess et al., 1999; Lacey et al., 1998). Injection of RANKL into normal mice induced systemic hypocalcaemia (Burgess et al., 1999). RANKL activates mature osteoclasts to resorb bone by inducing pseudopodial motility, spreading and actin ring formation (Burgess et al., 1999; Fuller et al., 1998; Udagawa et al., 1999). It was also reported that RANKL stimulated *cathepsin K* mRNA level in primary and cultured osteoclasts, which led to enhanced bone resorption (Corisdeo et al., 2001). RANKL enhances osteoclast survival by inhibiting apoptosis (Fuller et al., 1998; Jimi et al., 1999a; Udagawa et al., 1999).

Targeted deletion of RANKL in mice led to severe osteopetrosis with no osteoclasts and impaired tooth eruption (Kong et al., 1999b). Transgenic overexpression of RANKL in lymphocytes of RANKL-deficient mice only partially rescued the osteopetrosis. The mice exhibited sclerotic metaphyses, unerupted teeth and growth plate defects (Kim et al., 2000).

RANKL also plays an important role in the immune system. A soluble form of RANKL containing only the extracellualr domain activates c-Jun N-terminal kinase (JNK) specifically in T cells (Wong et al., 1997b). It also mediates survival of dendritic cells in the immune system (Anderson et al., 1997; Kong et al., 1999d; Wong et al., 1999b; Wong et al., 1997a). *RANKL* knockout mice have immunologic abnormalities including defects in early differentiation of T and B lymphocytes and a lack of lymph nodes (Kong et al., 1999b). The level of RANKL is increased in many immune diseases, such as rheumatoid arthritis (Kong et al., 1999a; Kong et al., 1999b). In

addition, sRANKL has been found in lymphocyte supernatants (Kong et al., 1999b). This may explain the bone loss seen in many diseases, e.g. rheumatoid arthritis, that involves the immune system.

### 1.5.2.3 OPG

OPG (osteoprotegrin)/ OCIF (osteoclastogenesis inhibitory factor) was identified by two groups (Simonet et al., 1997; Tsuda et al., 1997; Yasuda et al., 1998a). It was identified as a novel member of the TNF- receptor (TNF-R) superfamily during a sequencing project of rat intestinal cDNA (Simonet et al., 1997), and as a heparin-binding basic glycoprotein purified from conditioned medium of human embryonic lung fibroblasts (Tsuda et al., 1997). OPG contains no hydrophobic transmembrane-spanning sequence, indicating that it is a soluble receptor (Simonet et al., 1997; Tan et al., 1997). OPG is produced by most cell types in bone marrow microenvironment.

OPG acts as a decoy receptor for RANKL and inhibits osteoclastogenesis both *in vivo* and *in vitro* by preventing RANKL binding to RANK, the real receptor on osteoclast precursors (Lacey et al., 1998). OPG blocked osteoclast formation induced by RANKL-expressing stromal cell lines in the presence of 1, 25$(OH)_2D_3$, and inhibited 1, 25$(OH)_2D_{3,}$, PTH and IL-11 stimulated osteoclast formation in cocultures (Tsuda et al., 1997). OPG blocked RANKL-induced osteoclastogenesis in a dose-dependent manner *in vitro*. It suppressed ovariectomy-induced bone loss in rat, and injection of OPG into animals increased bone mass and suppresses osteoclastic bone resorption (Simonet et al., 1997; Yasuda et al., 1998a).

Overexpression of *OPG* in mice resulted in severe osteopetrosis due to an arrest of osteoclast differentiation, but with normal tooth eruption and bone elongation (Simonet et al., 1997). Detailed histological analysis showed that overexpression of *OPG* specifically inhibits osteoclast differentiation and activation at the endosteal bone surface, but not the periosteal bone surface (Min et al., 2000). Mice in which the *OPG* gene has been disrupted develop severe osteoporosis due to enhanced osteoclastogenesis (Bucay et al., 1998; Mizuno et al., 1998), which can be rescued by injection of recombinant OPG or transgenic overexpression of OPG (Min et al., 2000). Furthermore, *OPG* deficiency led to an increased vascular calcification in the aorta and renal arteries, a phenomenon also seen in human osteoporosis, suggesting a role of OPG in preventing these arteries from pathological calcification. Osteoblasts

from *OPG*-deficient mice had higher potential to support osteoclast differentiation *in vitro*, although the level of *RANKL* mRNA expression was similar to the wild-type (Udagawa et al., 2000).

In addition to its capacity to inhibit osteoclastogenesis, OPG inhibits osteoclastic bone resorption (Fuller et al., 1998; Hakeda et al., 1998) by disrupting the formation of F-actin rings (Burgess et al., 1999) and inhibits osteoclast survival *in vitro* (Lacey et al., 2000). In addition to RANK, OPG also binds to tumour necrosis factor-released apoptosis-inducing ligand (TRAIL) (Emery et al., 1998), which doesn't bind RANK. OPG inhibits TRAIL-induced apoptosis of Jurkat cells, and TRAIL blocks the anti-osteoclastogenic activity of OPG.

### 1.5.3 Regulatory factors for osteoclast formation and function

Most factors that regulate osteoclast formation and function act indirectly by binding to stromal cells and osteoblasts, which in turn modulate the expression of RANKL and OPG (Hofbauer et al., 1998; Horwood et al., 1998a; Udagawa et al., 1999; Yasuda et al., 1998b). Table 1.2 summarises the effect of hormones and cytokines on RANKL and OPG expression.

**Table 1.2 Factors that modulate RANKL and OPG expression**

| | RANKL | OPG | References |
|---|---|---|---|
| **Hormones** | | | |
| Dexamethasone | stimulate | inhibit | (Hofbauer et al., 1999a; Vidal et al., 1998) |
| Vitamin $D_3$ | stimulate | stimulate/inhibit | (Hofbauer et al., 1998; Horwood et al., 1998a) |
| PTH/PTHrP | stimulate | inhibit | (Yasuda et al., 1998b) |
| Estradiol | No change | stimulate | (Hofbauer et al., 1999b) |
| Prostaglandin E2 | stimulate | inhibit | (Brandstrom et al., 1998a; Murakami et al., 1998) |
| Glucocorticoid | stimulate | inhibit | (Huang et al., 2001) |
| **Cytokines** | | | |
| TNF-α | stimulate | stimulate | (Brandstrom et al., 1998b; Hofbauer et al., 1999c) |
| IL-1 | stimulate | stimulate | (Kwon et al., 1998; Vidal et al., 1998) |
| IL-6 | stimulate | ? | (Menaa et al., 2000) |
| IL-11 | stimulate | ? | (Ahlen et al., 2002) |
| IL-17 | stimulate | ? | (Kermanac et al., 2002) |
| **Growth factors** | | | |
| TGF-β | inhibit | stimulate | (Murakami et al., 1998; Takai et al., 1998) |
| BMP-2 | ? | stimulate | (Hofbauer et al., 1998) |

### 1.5.3.1 Calcitriol

Metabolites of vitamin $D_3$ are potent stimulators of osteoclastic bone resorption and osteoclast formation. The most active metabolite, $1,25(OH)_2D_3$, acts as a fusigen for committed osteoclast precursors (Kurihara and Roodman, 1990). Mice treated with $1,25(OH)_2D_3$ developed hypocalcaemia (Abe et al., 1989). On the other hand, mice lacking vitamin D receptor (VDR) developed normally up to 3 weeks, but exhibited a marked increase in osteoid tissues in the primary spongiosa at 7 weeks, with abundant osteoclasts present on bone surfaces. Spleen cells from *VDR*-/- mice were able to form osteoclasts when cultured with wild type osteoblasts in the presence of $1,25(OH)_2D_3$. In contrast, osteoblasts from *VDR*-/- mice failed to support osteoclast formation from wild-type spleen cells in the presence of $1,25(OH)_2D_3$, which could be rescued by PTH or IL-1, suggesting $1,25(OH)_2D_3$ regulates osteoclast development indirectly by osteoblasts and the actions of $1,25(OH)_2D_3$ on osteoclastic bone resorption can be compensated by other osteotropic factors (Takeda et al., 1999). The mouse *RANKL* promoter contains a vitamin D responsive element and transient transfection studies showed that treatment of ST2 stromal cells with $1,25(OH)_2D_3$ upregulates *RANKL* gene expression at the transcriptional level, suggesting that *RANKL* is a direct $1,25(OH)_2D_3$ target gene (Kitazawa and Kitazawa, 2002; Kitazawa et al., 1999). Yasuda et al. (Tsukii et al., 1998) reported that $1,25(OH)_2$ $D_3$ induced *RANKL* expression by osteoblasts. $1,25(OH)_2D_3$ also increased the expression level of *M-CSF* in human bone marrow cultures (Sarma and Flanagan, 1996). Furthermore, $1,25(OH)_2D_3$ enhances osteoclastic bone resorption stimulated by parathyroid hormone. However, it was also reported that $1,25(OH)_2D_3$ *in vivo* inhibited ovariectomy-induced bone resorption by suppressing osteoclasts formation (Sakai et al., 2001; Shibata et al., 2002).

It is unknown whether $1,25(OH)_2D_3$ acts on mature osteoclasts directly, although mature osteoclasts have been shown to express vitamin D receptors (Menaa and Roodman 1997). In murine stem cells, $1,25(OH)_2D_3$ downregulated *c-fms* (the receptor for M-CSF) expression in early monocytic precursors, but upregulated expression in more differentiated precursors (Perkins et al., 1995; Perkins and Teitelbaum, 1991).

### 1.5.3.2 Calcitonin

Calcitonin is a polypeptide hormone secreted by the parafollicular cells of the thyroid gland. It regulates calcium homeostasis by inhibiting osteoclastic bone resorption and by enhancing calcium excretion by the kidney (Warshawsky et al., 1980). Calcitonin inhibits both osteoclast formation and the bone-resorbing capacity of mature osteoclasts (Chambers et al., 1985b; Lee et al., 1995). The physiological effects of calcitonin are mediated by high affinity calcitonin receptors (CTR). Calcitonin acts directly on osteoclasts by binding to CTR, then activates adenylate cyclase activity and induces cAMP accumulation, resulting in immobilisation of the osteoclast and contraction of the osteoclast away from the bone surface (Chambers and Magnus, 1982; Gorn et al., 1995). Calcitonin also inhibits osteoclast secretory activity, particularly of TRAP (Yumita et al., 1991). It downregulates global transcriptional activity of resorbing osteoclasts *in vitro*, which was reversible (Boissy et al., 2002).

Osteoclasts continuously exposed to calcitonin can escape the effects of calcitonin (Wada et al., 1997). In neonatal mouse calvarial and adult bone marrow cultures, *CTR* mRNA expression was constitutively present and was markedly decreased after calcitonin treatment (Ikegame et al., 1996; Lee et al., 1995). The mechanism responsible for this ligand-induced desensitisation is unclear, but it is consistent with classical receptor desensitisation processes observed with other polypeptide hormones, such as PTH. It has been shown that calcitonin downregulated *CTR* gene expression on the surface of osteoclasts by a transcriptional mechanism (Inoue et al., 1999; Lee et al., 1995; Rakopoulos et al., 1995). Besides the inactivation effect on osteoclasts, calcitonin promotes osteoclast survival by delaying the onset of apoptosis (Selander et al., 1996).

### 1.5.3.3 Estrogen

Estrogen is one of the major inhibitors of osteoclast formation. In humans, loss of estrogen after menopause results in a progressive reduction of bone mass and causes osteoporosis. Estrogen replacement therapy improves postmenopausal osteoporosis (Jilka et al., 1992; Kimble et al., 1996; Pacifici, 1996). In rodents, osteoclastic bone resorption and osteoclast formation were increased after ovariectomy.

It is well known that estrogen deficiency induces bone resorption by enhancing osteoclast formation, reducing osteoclast apoptosis and increasing osteoclast activity

(Manolagas, 2000). Estrogen increases *OPG* expression, and decreases *M-CSF* and *RANKL* expression in osteoblastic cells (Hofbauer et al., 2000; Pacifici, 1996; Shevde et al., 2000). It suppresses RANKL-induced osteoclast formation through a repression in the level and functional activity of c-Jun (Shevde et al., 2000). In addition, estrogen inhibits osteoclastic bone resorption *in vivo* by downregulating the expression of proinflammatory cytokines, such as IL-6, IL-1, TNF-α and PGE2. Estrogen promoted osteoclasts apoptosis mediated by TGF-β in coculture system (Hughes et al., 1996). It was also reported that estrogen suppresses osteoclastogenesis by inhibiting T cell production of TNF-α in ovariectomised mice (Cenci et al., 2000).

Although osteoblasts are the effectors for estrogen action, Oursler and coworkers demonstrated that osteoclasts also contain estrogen receptors (Oursler et al., 1991). Estrogen inhibited osteoclast formation from rat haematopoietic precursors in the presence of M-CSF and RANKL, suggesting that estrogen has a direct effect on osteoclast precursors (Gyda et al., 2001).

#### 1.5.3.4 Interleukin 1 (IL-1)

Interleukin 1 (IL-1) is a cytokine produced by monocyte-macrophages and marrow stromal cells and osteoblasts. It is one of the most potent stimulators of bone resorption (Thomson et al., 1986). IL-1 induces bone resorption and osteoclast-like cell formation in murine and human marrow cultures (Gowen et al., 1983; Pfeilschifter et al., 1989).

IL-1 affects all stages of osteoclast development (Uy et al., 1995). It stimulates proliferation of osteoclast precursors (Pfeilschifter et al., 1989) and it induces the multinucleation of prefusion osteoclasts and bone-resorbing activity even in the absence of osteoblasts/stromal cells (Jimi et al., 1999b; Jimi et al., 1998). TNF-α-induced osteoclasts resorb bone only in the presence of IL-1 (Kobayashi et al., 2000). IL-1 enhances the survival of osteoclasts *in vitro* by activation of NF-κB (Jimi et al., 1999a; Jimi et al., 1998). Proteasome inhibitors, which inhibit NF-κB activation by preventing IκB degradation, and antisense oligonucleotides to NF-κB, suppressed IL-1α induced osteoclast survival. However, Miyazaki et al. reported that IL-1α supports osteoclast survival by inducing ERK activity, and stimulates resorption by NF-κB activation (Miyazaki et al., 2000). It has been reported that IL-1 can replace

RANKL to induce fusion, activation and survival, but not differentiation of osteoclasts (Suda et al., 1999).

IL-1 enhances osteoclastogenesis indirectly via osteoblastic cells (Thomson et al., 1986). It induces the expression of other cytokines, which stimulate osteoclast differentiation, such as IL-6, M-CSF and RANKL (Elias and Lentz, 1990; Felix et al., 1989; Kwon et al., 1998; Vidal et al., 1998). IL-1 is suggested to play an important role in several pathological conditions associated with increased bone loss, such as postmenopausal osteoporosis, rheumatoid arthritis, Paget's disease and tumours (Fried et al., 1989; Kitazawa et al., 1994; Mino et al., 1998; Pioli et al., 1989; Sato et al., 1989).

#### 1.5.3.5 Tumour Necrosis Factor-α (TNF-α)

Tumour Necrosis Factor-α (TNF-α) is a member of the tumour necrosis factor ligand superfamily. It is a multifunctional proinflammatory cytokine regulating cell proliferation, differentiation and apoptosis in various types of cells (Ashkenazi and Dixit, 1999). TNF-α plays an important role in host defence mechanisms, including the induction of macrophage cytocidal activity. It has been reported that TNF-α plays an important role in bone resorption of inflammatory bone diseases, such as periodontal disease (Abu-Amer et al., 1997) and rheumatoid arthritis (Keffer et al., 1991; Redlich et al., 2002). TNF-α also mediates bone loss in estrogen deficiency osteoporosis (Kimble et al., 1997; Kimble et al., 1996).

TNF-α affects osteoclasts both directly and indirectly. TNF-α stimulates osteoclast differentiation and function by inducing M-CSF and RANKL expression in osteoblasts (Hofbauer et al., 1999c; Yao et al., 2000). Thomson et al. showed that IL-1 or TNF-α stimulated bone resorption in a coculture system of mature osteoclasts and osteoblastic cells (Thomson et al., 1987). However, several recent studies showed that TNF-α stimulates osteoclastogenesis and activation directly. TNF-α augmented osteoclast formation induced by RANKL in a dose-dependent manner (Lam et al., 2000), and promoted actin ring formation and bone resorption of both primary osteoclasts and osteoclasts induced *in vitro* (Fuller et al., 2002). TNF-α and RANKL synergistically upregulated RANK expression in osteoclast precursors (Zhang et al., 2001). It was also reported that TNF-α stimulated bone marrow macrophages to differentiate into functional osteoclasts even in the absence of

RANKL (Kobayashi et al., 2000). The bone resorptive function of TNF-α-induced osteoclasts was enhanced by IL-1 without an increase in the number of osteoclasts (Azuma et al., 2000; Fuller et al., 2002). However, Kobayashi et al. reported that the bone resorption was induced only in the presence of IL-1 (Kobayashi et al., 2000). In contrast, Lam et al. showed that TNF-α alone failed to induce osteoclast formation at any concentration, but potentiated osteoclastogenesis from osteoclast precursors exposed to permissive levels of RANKL (Lam et al., 2000; Zhang et al., 2001).

TNF-α exerts its biological effects by binding to its two receptors present on osteoclast precursors: TNF receptor 1 (p55r) and 2 (p75r). P55r is the major form involved in osteoclast differentiation. The osteoclastogenesis induced by TNF-α was completely inhibited by an anti-p55r antibody and partially inhibited by an anti-p57r antibody (Azuma et al., 2000; Kobayashi et al., 2000). T cells from ovariectomised mice lacking TNF-α receptor p55 failed to augment RANKL-induced osteoclastogenesis (Cenci et al., 2000). Osteoclast formation induced by RANKL and the enhanced effect of TNF-α were both decreased in bone marrow cultures from p55, but not p75 knockout mice (Zhang et al., 2001). The activation of IκB, NF-κB, ERKs and c-Jun/AP-1 was reduced in p55 deficient precursors. Soluble TNF-α binds primarily to p55r, while membrane bound TNF-α targets both receptors (Abu-Amer et al., 2000). After interaction with the ligand, both receptors transduce intracellular signals and stimulate the proteolytic breakdown of IκB, which is a cytoplasmic inhibitor of NF-κB. The activated NF-κB is then translocated into the nucleus and induces the transcription of TNF-α responsive genes (Kruppa et al., 1992; Verma et al., 1995). It was also suggested that TRAF-6 is involved in TNF-α-induced osteoclastogenesis, as much less osteoclasts were induced from *TRAF-6*-deficient haematopoietic precursors in the presence of TNF-α (Kaji et al., 2001).

### 1.5.3.6 Interferon gamma (IFN-γ)

Interferon gamma (IFN-γ) is cytokine secreted by activated T cells. It is a potent inhibitor of bone resorption (Gowen and Mundy, 1986; Takahashi et al., 1986). Injection of IFN-γ suppresses the formation of osteoclasts *in vivo*, resulting in decreased plasma calcium concentrations (Tohkin et al., 1994). Mice lacking one of the IFN-γ receptor components (IFNGR1) (*IFN-γR*-/-) developed exacerbation of osteo-

clast formation and bone destruction. Collagen-induced arthritis in *IFN-γR-/-* mice developed earlier and had more severe bone destruction than normal mice (Manoury-Schwartz et al., 1997; Vermeire et al., 1997). IFN-γ directly suppresses both RANKL and TNF-α induced osteoclast formation and maturation (Fox and Chambers, 2000; Takahashi et al., 1986; Takayanagi et al., 2000). It primes macrophages for cytocidal activation by TNF-α (Fox et al., 2000).

The molecular mechanism by which IFN-γ acts on osteoclasts is not clear. The inhibitory effect of IFN-γ on osteoclastogenesis was completely abrogated in *STAT1-/-* mice, but not in *IRF-1-/-* mice suggesting that IFN-γ acts through the GAF (IFN-γ-activated factor, the active form of Stat1)-mediated IRF-1-independent gene induction pathway. The fact that TRAF-6 expression and RANKL-induced activation of NF-κB and JNK (see also below) in osteoclast precursors were markedly inhibited by IFN-γ, and osteoclast precursors overexpressing TRAF-6 were resistant to IFN-γ, suggests that TRAF-6 is one target gene of IFN-γ. It was suggested that IFN-γ exerts its inhibitory effect by activating the ubiquitin-proteasome pathway after binding to its receptor on osteoclast precursors, which causes the degradation of TRAF-6 (Takayanagi et al., 2000). Interestingly, the TRAF-6 degradation by IFN-γ requires RANKL signalling, since IFN-γ alone has no effect on TRAF6 expression. Thus, IFN-γ provides a negative link between T-cell activation and bone resorption.

### 1.5.3.7 Transforming growth factor-β (TGF-β) family

The transforming growth factor-β (TGF-β) superfamily, including TGF-βs, activins and bone morphogenetic proteins (BMPs), regulates cell proliferation, differentiation and apoptosis of many cell types (Josso and di Clemente, 1997; Moses and Serra, 1996). There are three TGF-β isoforms: TGF-β1, -β2 and -β3 expressed in mammalian tissues (Fox and Chambers, 2000). TGF-βs are secreted by osteoblasts and osteoclast progenitors, and are present in their latent forms within bone matrix (Kaneda et al., 2000; Robey et al., 1987; Sandberg et al., 1988; Seyedin et al., 1985). Active TGF-β is a 25 kD homodimer and contains nine cysteines which must be dissociated from a secreted latent complex to become biologically active (Seyedin et al., 1986).

It has been known for a long time that TGF-β is a potent inhibitor of osteoclastic bone resorption. It modulates osteoclastic bone resorption, migration and osteoclast differentiation in bone organ cultures (Dieudonne et al., 1991). Chenu et al. (Chenu et al., 1988) reported that TGF-β inhibits both the proliferation and fusion of human osteoclast precursors. TGF-β also increased apoptosis of osteoclasts induced in a coculture system in a dose-dependent manner (Hughes et al., 1996). However, recent experiments showed that the effects of TGF-β on osteoclastogenesis *in vitro* vary with the model employed. TGF-β enhanced osteoclast differentiation in the presence of IL-1 in a coculture system (Koide et al., 1999). It promotes osteoclast differentiation induced by sRANKL and M-CSF in the absence of stromal cells (Koseki et al., 2002), and suppresses apoptosis in osteoclasts (Fuller et al., 2000b)., consistent with the increase in cell size and multinuclearity (Sells Galvin et al., 1999). The stimulatory effect was only seen when TGF-β was added at early stages in the culture (Fuller et al., 2000b; Quinn et al., 2001). TGF-β also strongly promoted TNF-α induced osteoclast-like cell formation in the absence of RANKL *in vitro* (Fox et al., 2000; Quinn et al., 2001). As a macrophage deactivator, TGF-β may promote osteoclast formation by directing TNF-α activated precursors towards the osteoclast lineage (Fox et al., 2000; Fuller et al., 2000b).

TGF-β represents one growth factor that can either inhibit or stimulate osteoclast differentiation via different mechanisms. In the coculture system, TGF-β inhibits osteoclastogenesis by upregulating OPG expression and downregulating RANKL expression in osteoblastic and stromal cells (Murakami et al., 1998; Quinn et al., 2001; Takai et al., 1998). However, in the absence of osteoblasts/stromal cells, TGF-β may act directly on osteoclast progenitors (Sells Galvin et al., 1999), as osteoclast precursors express TGF-β receptors (Kaneda et al., 2000). Antibodies against TGF-β blocked osteoclastogenesis *in vitro* (Kaneda et al., 2000), suggesting that endogenous TGF-β is required for osteoclast differentiation.

TGF-β is one of the key factors involved in coupling bone formation to previous bone resorption. Bone matrix is one of the major storage sites for TGF-βs. During bone remodelling, TGF-β is released by bone lining cells and osteoclastic bone resorption, and modulates bone formation and resorption. Transgenic mice overexpressing cytoplasmically truncated type II TGF-β receptors using an osteocalcin

promoter had an age-dependent increase in bone mass with lower levels of osteolysis (Filvaroff et al., 1999). The defect was due to the imbalance between bone formation and bone resorption, as the rate of osteoblastic bone formation was not altered, but the number and activity of osteoclasts were decreased. On the other hand, transgenic mice overexpressing *TGF-β2* using an osteocalcin promoter developed age-dependent high turnover osteoporosis with increased osteoblastic matrix deposition and osteoclastic bone resorption (Erlebacher and Derynck, 1996). Detailed analysis showed that TGF-β2 directly stimulates osteoblast differentiation, while the increased mineral apposition rate depended on osteoclastic activity. Also, at sites undergoing active bone remodelling, the TGF-β2-induced increase in osteoblast differentiation was enhanced by osteoclast activity (Erlebacher et al., 1998).

Activin A is another member of TGF-β superfamily. It is a cytokine produced by bone marrow stromal cells, osteoblasts and osteoclasts and is abundantly present in bone matrix. Activin A enhances osteoclastogenesis induced by RANKL similarly to TGF-β *in vitro* (Fuller et al., 2000a).

The TGF-β superfamily acts through heteromeric complexes of type I and type II serine/threonine kinase receptors. The intracellular signalling downstream of these receptors is mediated by the Smad family. Smad2 and Smad3 are specifically involved in TGF-β and activin signalling pathways, and form heteromeric complexes with the common Smad, Smad4. Following nuclear translocation, Smads induce transcriptional activation of specific target genes through cooperation with other transcriptional factors (ten Dijke et al., 2000). TGF-β and activin A enhance RANKL induced osteoclast formation by upregulating JunB expression (Koseki et al., 2002). TGF-β may also synergistically increased the translocation of NF-κB into nuclei induced by RANKL in osteoclast precursors (Kaneda et al., 2000).

### 1.5.4 Role of bone marrow microenvironment in osteoclast development and function

It has been known for many years that stromal cells or osteoblasts are required for osteoclast differentiation from haematopoietic precursors both *in vivo* and *in vitro*. When osteoblastogenesis is absent, as in *Cbfa-1* knockout mice, osteoclastogenesis is impaired (Ducy et al., 1997; Komori et al., 1997). Osteoclast precursors recognise signals expressed by osteoblasts/stromal cells in response to osteotropic

factors and differentiate into osteoclasts. Cell-to-cell contact between osteoblasts/stromal cells and haematopoietic cells was indispensable for osteoclast differentiation (Takahashi et al., 1988b), since it was reported that when osteoclast precursors and stromal cells were separated by a semi-permeable membrane in the coculture system, no osteoclasts formed, and most of the cells were F4/80-positive macrophage–like cells (Tsurukai et al., 1998). Now it is clear that two molecules expressed in these cells are essential and sufficient for osteoclastogenesis: M-CSF and RANKL (Kodama et al., 1991; Yasuda et al., 1998b). During osteoblast differentiation, RANKL expression remains constant, while OPG expression is increased after the onset of mineralisation resulting in a decreased RANKL/OPG ratio, suggesting immature osteoblasts have higher osteoclastogenic potential than mature osteoblasts (Thomas et al., 2001).

In addition to their expression of M-CSF and RANKL, osteoblasts/stromal cells are target cells for osteotropic hormones and cytokines to induce osteoclast development. Osteotropic factors *e.g.* PTH, 1, 25$(OH)_2D_3$, and prostaglandin $E_2$ act indirectly by binding to osteoblasts or marrow stromal cells, which in turn induce upregulation of RANKL expression and downregulation of OPG. RANKL then binds the RANK receptor on osteoclast precursors and induces osteoclast formation (Atkins et al., 2000; Hofbauer and Heufelder, 1998; Itoh et al., 2000; Yasuda et al., 1998b) (see also above). McSheehy and Chambers showed that when the stimulating factors were added directly to isolated osteoclasts, there was no increase in bone resorption, but when these agents were added to the osteoblasts and conditioned medium was applied, bone resorption was increased (McSheehy and Chambers, 1986).

Osteoblasts/bone marrow stromal cells are also required for osteoclast function. Highly purified osteoclasts cultured on dentine slices failed to form resorption pits, and the resorptive capability of these purified osteoclasts was restored when calvarial osteoblasts were added. Several stromal cell lines such as KS-4, MC3T3-G2/PA6, and ST2 also potentiated pit-forming activity of enriched osteoclasts. When cell-to-cell contact between osteoblasts/stromal cells and osteoclasts was prevented, osteoclasts failed to form resorption pits (Jimi et al., 1996; Suda et al., 1997).

Other cell types in the bone marrow also have the ability to support osteoclast differentiation. The expression of the factors regulating osteoclast differentiation is

not restricted to the cells of the osteoblastic lineage. Although similar to the *Cbfa-1* knockout mice, *Osx* null mice developed only cartilagenous skeleton, functional osteoclasts were still present in the mutant long bone (Nakashima et al., 2002), suggesting that other cells are synthesising the appropriate osteoclastogenic factors.

In pathological conditions, immune cells, especially activated T cells, in the bone marrow contribute to the regulation of bone resorption. Immune cells produce a variety of cytokines, including IL-1, TNF-α, IL-6 and prostaglandins, which have been shown to stimulate osteoclast development and activation (Grcevic et al., 2001)(see also below). Evidence also showed that B-lymphoid lineage cells expressed RANKL and supported osteoclast formation *in vitro*. A decrease in B-lymphoid cells in *klotho* mutant mice (*KL*-/-) led to a decrease in osteoclast numbers, and osteoclast formation from *KL*-/- bone marrow cells are reduced. It was suggested that B-lymphocytes might be involved in the pathophysiology of bone disorders, such as osteoporosis (Manabe et al., 2001).

T lymphocytes have both stimulatory and inhibitory effects on osteoclastogenesis. RANKL was first identified as TNF-related activation-induced cytokine (TRANCE), a T cell product upregulated after activation (Wong et al., 1997a). During inflammation, activated T cells produce RANKL promoting osteoclastic bone resorption and bone loss, but at the same time, T cells also release IFN-γ and IFN-β (see also below) limiting bone destruction (Kong et al., 1999b; Takayanagi et al., 2000). Activated T cells play a critical role in the bone destruction which occurs in inflammatory diseases, such as rheumatoid arthritis, and periodontal disease (Kong et al., 1999c). T cells also play an important role in estrogen deficiency induced bone loss (Roggia et al., 2001). For example, ovariectomy induced rapid bone loss was absent in T-cell deficient mice.

In conclusion, osteoclastogenesis is tightly controlled by osteoblasts/stromal cells in bone marrow microenvironment. However, in pathological conditions, other cell types such as lymphocytes, also play an important role in regulating osteoclast formation and function which leads to the imbalance between bone formation and resorption, and subsequently causes bone destruction.

## 1.6 Model systems for studying osteoclast formation and bone resorption

In order to study the biological and molecular mechanisms involved in osteoclast differentiation and bone resorption, various model systems, both *in vivo* and *in vitro*, have been established for different purposes.

Osteopetrotic animal models generated either by targeted disruption of transcription factors and signalling molecules, or by naturally-occurring mutations, have greatly contributed to the understanding of osteoclast biology. Recent studies in knockout and transgenic mice have identified a number of genes that are required for osteoclast formation and activation, and these are summarised in Table 1.3.

**Table 1.3 Animal models of osteopetrosis**

| | Mutated gene | No. OC | No. Mac | RB | BMT | References |
|---|---|---|---|---|---|---|
| **Spontaneous mutation** | | | | | | |
| Osteopetrosis mouse (op/op) | M-CSF | ↓↓↓ | ↓↓↓ | N | no cure | (Yoshida et al., 1990) |
| Microphthalmia mouse (mi/mi) | mi gene | N | nr | A | cure | (Hodgkinson et al., 1993) |
| Osteosclerosis mouse (oc/oc) | OC116 | ↓ | nr | A | No cure | (Marks et al., 1985) |
| Grey-lethal mouse (*gl/gl*) | Chromosome 10 | ↓ | E↓ | N | cure | (Rajapurohitam et al., 2001) |
| Toothless rat (tl/tl) | ? | ↓↓↓ | ↓↓↓ | N | no cure | (Marks et al., 1993) |
| Incisor absent rat (ia/ia) | ? | N | nr | A | cure | (Reinholt et al., 1999) |
| Osteopetrotic rat (op/op) | Chromosome 10 | ↓ | nr | PD | cure | (Marks and Popoff, 1989) |
| Microphthal mia Blanc (*mib*) rat | *mib* | ↓ | nr | PD | - | (Cielinski and Marks, 1995) |
| Osteopetrotic (*os*) rabbit | ? | ↓ | nr | nr | no cure | (Lenhard et al., 1990) |
| **Induced mutation** | | | | | | |
| *c-src* knockout mouse | c-src | N | N | A | cure | (Boyce et al., 1992) |
| *c-fos* knockout mouse | c-fos | A | ↑ | - | cure | (Wang et al., 1992) |
| *NFκB* p50/p55 knockout mouse | NFκB p50/p55 | ↓↓↓ | ↑ | - | cure | (Franzoso et al., 1997) |
| PU.1 knockout mouse | PU.1 | A | ↓ | - | cure | (Tondravi et al., 1997) |

Abbreviations: A: absent; BMT: bone marrow transplantation; E↓: elicit Macrophage reduced; N: normal; nr: not reported; No.Mac: number of macrophages; No.OC: number of osteoclasts; PD: poorly developed; RB: ruffled border; ↑: elevated;↓: reduced; ↓↓↓: severely reduced.

### 1.6.1 Mouse osteopetrosis models

#### 1.6.1.1 PU.1

The transcription factor PU.1 is encoded by the *Spi-1/Sfp-1* gene, a member of the *ets* proto-oncogene family. It is expressed specifically in the monocytic and B lymphoid lineages (Klemsz et al., 1990). The expression of *PU.1* mRNA increased with the induction of osteoclastogenesis by $1,25(OH)_2$-$D_3$ or Dexamethasone (Tondravi et al., 1997). PU.1 regulates monocyte/macrophage differentiation, survival and proliferation by controlling the expression of *c-fms* (Klemsz et al., 1990). *PU.1* expression progressively increased as marrow macrophages assumed the osteoclast phenotype *in vitro*. Mice deficient in *PU.1* lack not only macrophages but also osteoclasts, leading to the development of severe osteopetrosis (Tondravi et al., 1997), suggesting that this transcription factor regulates the initial stages of myeloid differentiation. Bone marrow transplantation rescued the osteopetrosis with complete restoration of osteoclast and macrophage differentiation (Tondravi et al., 1997), suggesting that the defect is intrinsic to haematopoietic lineage. *In vitro* studies showed that *PU.1*-/- haematopoietic progenitors committed to the monocyte lineage, but were unable to develop into mature macrophages in the absent of PU.1 (Klemsz et al., 1990). To date, PU.1 is the earliest known marker of osteoclast differentiation as it controls early myeloid cell differentiation, which includes macrophages and osteoclasts.

#### 1.6.1.2 c-Src

The *c-src* proto-oncogene encodes a non-receptor tyrosine kinase. Src kinase is activated by a variety of signalling pathways including stimulation of growth factor receptors, G protein-coupled receptors, oxidative and UV stress, and integrin-mediated signal transduction (Brown and Cooper, 1996). The *c-src* product, $pp60^{c\text{-}src}$, is highly expressed in osteoclasts and is primarily localised on ruffled border membranes and vacuoles (Horne et al., 1992; Tanaka et al., 1992). The expression of $pp60^{c\text{-}src}$ protein and src kinase activity were upregulated in bone marrow cells after culturing with $1,25(OH)_2D_3$ (Horne et al., 1992). Targeted gene disruption of c-Src induced osteopetrosis in mice demonstrating that *c-src* plays a critical role in osteoclast activation. Osteoclast formation was normal in *c-src* knockout mice, however,

those osteoclasts couldn't form ruffled borders, therefore they are unable to resorb bone (Boyce et al., 1992). The distribution of the actin ring in *c-src-/-* osteoclasts was markedly different from the wild-type, with no peripheral podosome arrangement (Schwartzberg et al., 1997). TRAP positive osteoclasts could be induced from *c-src* deficient spleen cells cocultured with wild-type osteoblasts, but they were smaller and not functional. Osteoblasts from *c-src-/-* mice supported osteoclast formation similarly to wild-type osteoblasts. Fetal liver transplantation rescued osteopetrosis and tooth eruption (Lowe et al., 1993), suggesting the defect is intrinsic in the osteoclast lineage. Moreover, transgenic mice overexpressing chicken *c-src* from the TRAP promoter rescued the osteopetrosis and the lack of tooth eruption in *c-src-/-* mice, and osteoclasts developed normal ruffled border. Overexpression of kinase-defective alleles of *c-src* also reduced osteopetrosis and partially rescued the organisation of cytoskeleton of osteoclast, suggesting a kinase-independent function of *c-src* in osteoclast signalling pathways (Schwartzberg et al., 1997).

Recent studies showed that the tyrosine phosphorylation of c-Cb1, a substrate of c-Src, and PYK2, a major adhesion-dependent tyrosine kinase in osteoclasts, are markedly reduced in osteoclasts from *c-src* knockout mice, suggesting these two factors act downstream of c-Src in regulating osteoclast activation (Duong et al., 1998; Tanaka et al., 1996).

#### 1.6.1.3 Nuclear Factor-κB (NF-κB)

Nuclear Factor-κB (NF-κB) is another important transcription factor involved in osteoclast differentiation and function. NF-κB is a dimer composed of various combinations of proteins: p50 (NFKB1), p52 (NFKB2), p65 (Rel A), Rel B and c-Rel (Verma et al., 1995). These proteins are all related by the Rel homology domain that contains the DNA-binding motif. NF-κB dimers are generally retained in the cytoplasm by association with the inhibitory IκB proteins. In response to extracellular signals that lead to cell growth, differentiation, inflammatory responses, apoptosis, and neoplastic transformation, IκB is phosphorylated by specific IκB kinases and proteolytically degraded. This allows NF-κB to translocate to the nucleus to regulate the transcription of target genes.

Inhibitors of NF-κB inhibited osteoclastic bone resorption *in vitro* in a dose dependent manner (Hall et al., 1995b). Single deletion of either the p50 or p52 subunits didn't induce any developmental defects, although p50-/- mice had altered immune responses (Sha et al., 1995). In contrast, mice deficient in both the p50 and p52 subunits of *NF-κB* develop severe osteopetrosis caused by the arrested generation of osteoclasts from macrophages (Franzoso et al., 1997; Iotsova et al., 1997b). Occasionally, one or two weakly TRAP positive osteoclasts were present in the mutant long bones. The number of macrophages in bone marrow was increased, suggesting *NF-κB* acts at a point in osteoclast lineage very close to *c-fos* (see also section 1.7.3.1 below). Furthermore, the double knockout mice failed to generate mature B cells and exhibited defective thymus and spleen structure (Franzoso et al., 1997). Wild-type fetal liver or bone marrow transplantation rescued the defect in osteoclastogenesis and resulted in tooth eruption (Iotsova et al., 1997a). Osteoblasts from *NF-κB* double knockouts supported osteoclast differentiation from wild-type precursors, while mutant spleen cells failed to form osteoclasts in coculture with wild-type osteoblasts suggesting that the defect resides within osteoclast lineage.

It was demonstrated that NF-κB is a key downstream mediator of RANKL- and cytokine-induced osteoclastogenesis, such as IL-1, TNF-α and IL-6 (Beg and Baltimore, 1996; Siebenlist et al., 1994). More RANK-expressing osteoclast/macrophage precursors were present in *NF-κB* double knockout spleen, but they were unable to differentiate into mature osteoclasts. IL-1, TNF-α, M-CSF, and IL-6 plus soluble IL-6 receptor couldn't rescue the osteoclast defect (Xing et al., 2002). NF-κB also regulates osteoclast survival, as inhibitors of NF-κB induced apoptosis of mature osteoclasts isolated from rabbits and inhibited bone resorption activity (Ozaki et al., 1997). Antisense oligonucleotides to p65 or p50 subunits of *NF-κB* induced apoptosis in isolated osteoclasts by preventing translocation of *NF-κB* from the cytoplasm to the nucleus (Jimi et al., 1998).

#### 1.6.1.4 c-Fos

*C-fos,* a proto-oncogene normally associated with osteosarcomas, also appears to be a key regulator of osteoclast differentiation. A detailed review of the role of *c-fos* is found in section 1.7.3 below.

### 1.6.1.5 Microphthalmia (mi)

The *microphthalmia* (*mi*) gene encodes a Myc-related basic helix-loop-helix leucine-zipper-type (b-HLH-ZIP) protein, which belongs to a family of transcription factors including Mitf (*microphthalmia* transcription factor), TFE3, TFEB, and TFEC. *Mitf* is expressed in osteoclast progenitors and its expression is upregulated by M-CSF (Kawaguchi and Noda, 1998; Kawaguchi and Noda, 2000). Cathepsin K has recently been identified as a transcriptional target of Mitf (Motyckova et al., 2001).

It was reported that *mi* plays an important role in the fusion of osteoclast precursors. Mice deficient in the *mi* gene, called *mi/mi* mice, developed osteopetrosis with mononuclear osteoclast-like cells present, and had a defect in pigment cells and mast cells (Hodgkinson et al., 1993). Macrophages from *mi* mice have defects in phagocytic recognition and chemotactic responsiveness (Chambers and Loutit, 1979; Minkin, 1981). The osteopetrosis can be cured by transfusion with spleen cells from wild-type mice (Hodgkinson et al., 1993; Marks and Walker, 1981; Steingrimsson et al., 1994). When spleen cells from *mi/mi* mice were cultured with ST2 stromal cells, osteoclastogenesis was significantly reduced compared with wild-type spleen cells, while osteoblastic cells from *mi/mi* mice supported osteoclast differentiation *in vitro* similarly to the wild-type, suggesting that the defect of osteoclastogenesis in the absence of *Mitf* is due to the haematopoietic precursors rather than the stromal cells (Kawaguchi and Noda, 2000). Mitf is a target for the RANKL signalling pathway in osteoclast differentiation, and phosphorylation of Mitf increases the expression of osteoclast specific genes (Mansky et al., 2002). PU.1 and Mitf interact with each other in regulating osteoclast differentiation. Mice heterozygous for either the *mi* allele (*Mitf/mi*) or the *PU.1* allele (*PU.1*+/-) developed normal bone structure, while double heterozygous mice (*Mitf/mi/ PU.1*+/-) developed osteopetrosis at birth. The morphology and number of osteoclasts were normal, suggesting that the defect was in the later stage of osteoclastogenesis or in mature osteoclasts. The osteopetrosis resolved with age (Luchin et al., 2001).

### 1.6.1.6 Grey-lethal (*gl*) mouse

The *gl* mouse is the mutant that most closely resembles the severe human autosomal-recessive form of this hereditary defect. It has defect in chromosome 10. Bone

marrow transplantation could rescue the osteopetrosis, suggesting it's an osteoclast autonomous defect. Histological analysis showed numerous multinucleated osteoclasts present in the long bone, but they are dysfunctional. The *gl* osteoclasts demonstrated a defective cytoskeletal reorganisation and an underdeveloped ruffled border. Primary osteoclasts isolated from mutant mice showed significantly reduced resorptive activity *in vitro* (Rajapurohitam et al., 2001).

### 1.6.2 Rat osteopetrosis models

Similarly to mouse mutants, there are naturally occurring osteopetrotic models in rats, and these are also summarised in Table 1.3.

#### 1.6.2.1 Toothless *(tl)*

Toothless (*tl*) is an osteopetrotic mutation in rat. The *tl* rats developed generalised skeletal sclerosis with few osteoclasts, monocytes and macrophages, and a lack of tooth eruption, which couldn't be corrected by bone marrow transplantation (Marks et al., 1993). It has been suggested that the phenotype in *tl* mice is due to a deficiency in biological active M-CSF. Treatment of *tl* rats with human recombinant M-CSF reduced osteosclerosis and normalised osteoblastic gene expression. Although the number of osteoclasts increased significantly after treatment, compared to the untreated wild type mice, the osteoclast number and marker gene expression remained lower than untreated wild-type (Marks et al., 1993; Wisner-Lynch et al., 1995). M-CSF treatment also promoted angiogenesis in *tl* rats (Aharinejad et al., 1995), suggesting that M-CSF plays an important role in angiogenesis in rats.

#### 1.6.2.2 Incisor-absent (*ia*)

The incisor-absent (*ia*) rat developed osteopetrosis which could be cured by bone marrow transplantation (Marks, 1976). The fact that metatarsals from mutant rats developed a bone marrow cavity when cultured with wild-type spleen cells (Lenhard et al., 1990), suggests that the primary defect is intrinsic to the osteoclast lineage. Histological analysis revealed that the number of osteoclasts was increased in *ia* mutant rats compared to their wild-type littermates, but these osteoclasts couldn't form ruffled borders and exhibited extended clear zones, therefore failing to resorb bone (Marks, 1973). Immunocytochemistry showed that the expression level of $\alpha_V\beta_3$ integrin in the mutant osteoclasts was increased, and the *osteopontin* mRNA level

was upregulated in the mutant long bones and was homogenously distributed at the surface facing osteoclasts (Reinholt et al., 1999).

### 1.6.2.3 Osteopetrosis (*op*)

The osteopetrosis (*op*) in rats is a spontaneous lethal mutation, which can be cured by wild-type bone marrow transplantation. In *op* rats, the number of osteoclasts is significantly reduced, and they are larger and more vacuolated compared to the wild-type osteoclasts. The mutant osteoclasts form a ruffled border and clear zone, but their ability to break and excavate bone surfaces is impaired (Marks and Popoff, 1989).

### 1.6.2.4 Microphthalmia Blanc (*mib*)

Microphthalmia Blanc (*mib*) is a spontaneous osteopetrotic mutation in rats. The mutant rats were born with osteosclerosis, which was improved with age (Moutier et al., 1989). The mild transient osteopetrosis in the *mib* rat is caused by neonatal reductions in osteoclast number and function (Cielinski and Marks, 1994). The ruffled border was absent or poorly developed in neonatal mutant osteoclasts, and the expression of osteoclast marker genes such as CAII and TRAP were significantly reduced. The defects restored spontaneously within a month (Cielinski and Marks, 1995). Bone formation is not affected.

### 1.6.3 Rabbit osteopetrosis (*os*)

Osteopetrotic rabbits (*os*) developed skeletal deformities, abnormalities in osteoclasts and osteoblasts, aberrations in tooth development and eruption and bone matrix formation. This mutation is not cured by wild-type bone marrow transplantation, suggesting the reduced bone resorption is due to the defects in skeletal microenvironment. Mutant metatarsals cocultured with wild-type spleen, liver and bone marrow cells failed to develop bone marrow cavity, while the wild-type metatarsals could. The number and activity of osteoclasts induced from mutant culture were reduced (Lenhard et al., 1990). This mutant is also summarised in Table 1.3.

### 1.6.4 Culture systems

*In vitro* systems have been used to identify osteoclast precursors, and to characterise factors that affect osteoclast formation and activity. These are summarised in the following sections.

#### 1.6.4.1 Primary osteoclast isolation

Chambers et al. developed a technique whereby primary osteoclasts were isolated form neonatal rabbit long bones, and these cells were able to resorb bone *in vitro* (Chambers et al., 1984). This technique represents a model system with which to assess the direct effects of hormones, cells and substrate composition on the induction, stimulation and inhibition of osteoclastic bone resorption and to investigate the mechanism by which cells degrade extracellular matrix.

#### 1.6.4.2 Organ culture system

Long bones, fetal calvaria or metatarsals from rat and mouse have been widely used to examine osteoclast activity (Burger et al., 1986; Pfeilschifter et al., 1988; Smith et al., 1987; Weir et al., 1996). Bone organs were cultured with different growth factors or conditioned medium *in vitro*, and osteoclastic bone resorption was measured by histology or $^{45}Ca$ release. This system is particularly useful for evaluating the effects of osteotropic factors on osteoclastic bone resorption.

#### 1.6.4.3 Coculture system

Burger et al. developed a coculture system to investigate osteoclast formation *in vitro*, in which murine marrow cells were cultured with stripped fetal bone rudiments devoid of osteoclast progenitors (Burger et al., 1982). This system provided important information on the ontogeny of osteoclasts and differentiation of osteoclast precursors into bone-resorbing osteoclasts (Scheven et al., 1986; Thesingh, 1986). Subsequently, Takahashi et al. developed an efficient mouse marrow coculture system. Numerous TRAP positive multinucleated cells were generated when bone marrow cells were cultured with mouse osteoblastic cells in the presence of $1,25(OH)_2D_3$ or PTH (Takahashi et al., 1988b). It was found later that spleen cells can substitute for bone marrow cells as a source of osteoclast precursors in this system (Udagawa et al., 1989). This system is useful for investigating both osteoclast formation and bone resorption, and is widely adapted by many groups.

### 1.6.4.4 Stroma-free cell culture system

Although bone marrow/spleen coculture system closely resembles *in vivo* osteoclastogenesis, the presence of osteoblasts/stromal cells mixes up the direct and indirect effects of osteotropic factors. After the discovery of RANKL, this problem has been solved by culturing haematopoietic precursors, from bone marrow or spleen, with M-CSF and RANKL in the absence of stromal cells (Fox and Chambers, 2000; Udagawa et al., 1999). This simplified culture system provides opportunity to study osteoclast formation and function without the influence of osteoblastic cells, and is most widely used nowadays.

Recently, a number of groups established a culture system for the development of osteoclasts from pluripotent ES (embryonic stem) cells, permitting the whole process of osteoclastogenesis to be assessed starting from single ES cells (Hemmi et al., 2001; Yamane et al., 1997).

### 1.6.4.5 Human osteoclast generation

Functional multinucleated osteoclasts were successfully generated from human bone marrow cells, peripheral blood mononuclear cells (PBMCs) or tumour cells cultured with osteoblastic cell lines in the presence of $1,25(OH)_2D_3$ and M-CSF (Fujikawa et al., 1996; MacDonald et al., 1987; Quinn et al., 1998b), which permits studies on osteoclast formation in patients with metabolic bone disease. After the identification of RANKL, osteoclasts could be generated from human PBMCs in the absence of osteoblasts/stromal cells (Lader et al., 2001; Matsuzaki et al., 1998; Quinn et al., 1998a). By using this stroma-free culture system, human circulating osteoclast precursors were purified as CD14 (+) cells, which express RANK and could give rise to functional osteoclasts in the presence of M-CSF and RANKL (Nicholson et al., 2000). This model is particular helpful for analysing the pathogenesis and treatment of metabolic bone diseases, such as rheumatoid arthritis (RA) and bone tumours, and is widely used in clinical research.

Taken together, the *in vitro* culture systems described above are selectively used for different purposes. Among these, the stroma-free culture system is employed by many laboratories as it is particularly helpful in exploring the effect of osteotropic factors on osteoclasts and their precursors, and dissecting the molecular mechanisms of osteoclast differentiation from haematopoietic stem cells.

## 1.7 AP-1 family in bone biology

AP-1 (activator protein 1) is a transcription factor family, which includes several Fos-related (c-Fos, FosB, Fra-1 and Fra-2) and Jun-related (c-Jun, JunB, JunD) proteins. The AP-1 family genes share a hydrophobic leucine zipper domain that mediates protein dimerisation, and a basic region that mediates DNA binding. Transcriptional regulation by AP-1 members involves formation of homodimers and heterodimers between Jun and Fos members, or with Jun dimerisation partners (JDP1 and JDP2) and ATF/CREB family members, and subsesquently binding of DNA at AP-1 consensus sequences in regulatory regions of target genes (Angel and Karin, 1991; Shaulian and Karin, 2001).

AP-1 genes are important mediators of signal transduction, cellular transformation, cell proliferation and differentiation, maturation, apoptosis of various cell types. The role of AP-1 family members in embryonic and postnatal development have been identified by genetically modified mice models. Tables 1.4 and 1.5 summarise the consequences of targeted gene disruption, and of transgene overexpression in mice (Adapted from Jochum et al., 2001; Wagner, 2002).

**Table 1.4 AP-1 knock out mice**

| | Phenotype | Affected organs/ cell types | References |
|---|---|---|---|
| **Fos proteins** | | | |
| c-Fos | Osteopetrosis | Bone, osteoclasts | (Johnson et al., 1992; Wang et al., 1992) |
| FosB | Nurturing defect | Brain, hypothalamus | (Brown et al., 1996; Gruda et al., 1996) |
| Fra-1 | Embryonic lethality | Extraembryonic tissue, placenta | (Schreiber et al., 2000) |
| Fra-2 | Lethal at birth | Not published | (Wagner, 2002) |
| **Jun proteins** | | | |
| c-Jun | Embryonic lethality | Liver, hepatoblasts<br>Heart, outflow tract | (Hilberg et al., 1993; Johnson et al., 1993) |
| JunB | Embryonic lethality | Extraembryonic tissue, placenta | (Schorpp-Kistner et al., 1999) |
| JunD | Male sterility | Testis, spermatogenesis | (Thepot et al., 2000) |

**Table 1.5 AP-1 transgenic mice**

| | Promoters | Phenotype | Affected organ/cell types | References |
|---|---|---|---|---|
| **Fos proteins** | | | | |
| c-Fos | $H2k^b$ | Osteosarcoma | Bone, osteoblasts | (Grigoriadis et al., 1993) |
| | (chimaeric) | Chondrosarcoma | Bone, Chondrocytes | (Wang et al., 1991) |
| FosB | $H2k^b$ | Normal | | (Grigoriadis et al., 1993) |
| ΔFosB | TCRb | Impaired T cell differentiation | Thymus,immature thymocytes | (Carrozza et al., 1997; Sabatakos et al., 2000) |
| | NSE | osteosclerosis | Bone, osteoblasts | (Jochum et al., 2000) |
| Fra-1 | $H2k^b$ | | | |
| Fra-2 | CMV | Ocular malformation | Eye, anterior eye structure | (McHenry et al., 1998) |
| **Jun protein** | | | | |
| c-Jun | $H2k^b$ | Normal | | (Grigoriadis et al., 1993) |
| JunB | Ubiquitin C | Normal | | |
| | CD4 | Enhanced Th2 maturation | Thymus, CD4 thymocytes | (Li et al., 1999a; Schorpp et al., 1996) |
| JunD | Not reported | | | |

### 1.7.1 Molecular biology of c-Fos

The c-Fos oncoprotein is one of the major components of the AP-1 family. The *c-fos* proto-oncogene is the cellular homologue of *v-fos*, which is the transforming gene identified originally in the FBJ- and FBR-murine sarcoma viruses (MSVs). The mature *c-fos* mRNA transcript of 2.2kb encodes a 380-amino acid phosphoprotein that undergoes extensive posttranslational modification and has an apparent molecular mass of 55kDa. Due to its rapid expression in response to mitogenic stimulation, *c-fos* is classed as an immediate early gene and has been implicated in normal cellular growth control.

During early mouse development, *c-fos* is expressed in extraembryonic tissues (Muller et al., 1983), while expression during late embryonic development was restricted to the growth regions of foetal bone (De Togni et al., 1988; Dony and Gruss, 1987; Sandberg et al., 1988). In adult tissues, *c-fos* expression was detected in skeletal/haematopoietic tissues, including epiphyseal chondrocytes, osteoclasts, calcified trabeculae composed of hypertrophic cartilage and newly deposited bone, periosteal bone, and some cells of myeloid and lymphoid lineages of the marrow (Lord et al., 1993; Sandberg et al., 1988; Smeyne et al., 1992; Sunters et al., 1998). Fig.1.8 shows the role of c-Fos on bone cell biology (Adapted from Grigoriadis et al., 1995).

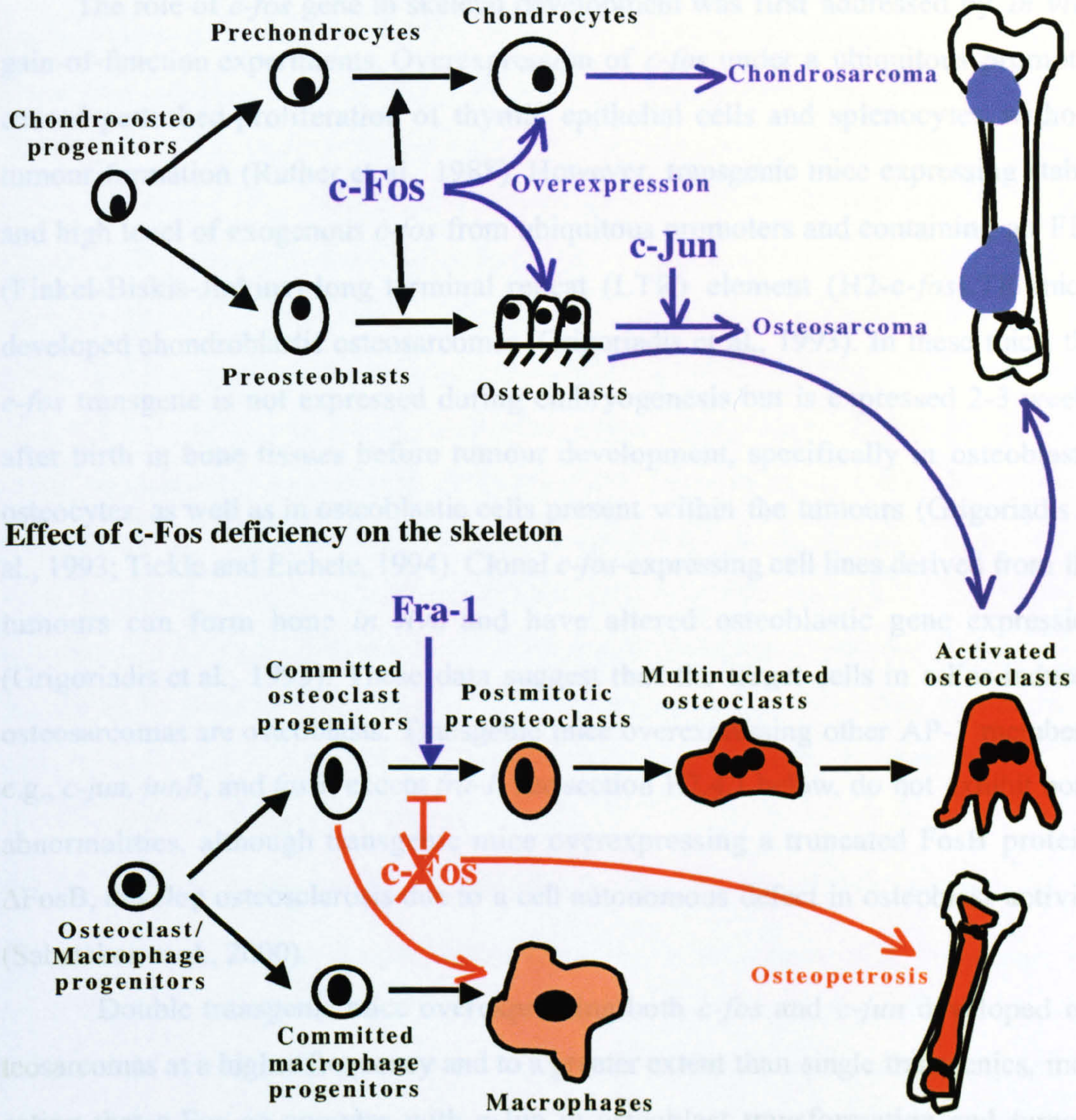



**Fig 1.8 The effect of overexpression and deletion of c-Fos on bone cells.** Top panel: overexpression of c-Fos in transgenic and chimaeric mice leads to the transformation of osteoblasts and chondrocytes, which results in the formation of osteosarcomas and chondrosarcomas. c-Jun co-operates with c-Fos in osteoblast transformation and tumour formation. Overexpression of c-Fos also enhances osteoclast formation and activation, which in turn plays an important role in tumour progression and tumour remodelling. Bottom panel: Deletion of c-Fos leads to a block in osteoclast differentiation and an increase in macrophage numbers, which results in the development of osteopetrosis. Overexpression of *fra-1* can rescue the defect of osteoclastogenesis in the absence of c-Fos. In both top and bottom panels, the role of endogenous c-Fos is represented by black arrows, the roles of the c-Fos, c-Jun and Fra-1 transgenes are represented by blue arrows and the consequences of c-Fos deletion is represented by red arrows.

### 1.7.2 Effects of *c-fos* on the osteoblast/chondrocyte lineage

#### 1.7.2.1 Transgenic mice overexpressing *c-fos* develop osteosarcoma

The role of *c-fos* gene in skeletal development was first addressed by *in vivo* gain-of-function experiments. Overexpression of *c-fos* under a ubiquitous promoter caused perturbed proliferation of thymic epithelial cells and splenocytes without tumour formation (Ruther et al., 1988). However, transgenic mice expressing stable and high level of exogenous *c-fos* from ubiquitous promoters and containing an FBJ (Finkel-Biskis-Jinkins)-long terminal repeat (LTR) element (H2-c-*fos*LTR mice) developed chondroblastic osteosarcomas (Grigoriadis et al., 1993). In these mice, the *c-fos* transgene is not expressed during embryogenesis but is expressed 2-3 weeks after birth in bone tissues before tumour development, specifically in osteoblasts, osteocytes, as well as in osteoblastic cells present within the tumours (Grigoriadis et al., 1993; Tickle and Eichele, 1994). Clonal *c-fos*-expressing cell lines derived from the tumours can form bone *in vivo* and have altered osteoblastic gene expression (Grigoriadis et al., 1993). These data suggest that the target cells in c-Fos-induced osteosarcomas are osteoblasts. Transgenic mice overexpressing other AP-1 members, e.g., *c-jun, junB*, and *fosB*, except *fra-1,* see section 1.7.4.1 below, do not exhibit bone abnormalities, although transgenic mice overexpressing a truncated FosB protein, ΔFosB, develop osteosclerosis due to a cell autonomous defect in osteoblast activity (Sabatakos et al., 2000).

Double transgenic mice overexpressing both *c-fos* and *c-jun* developed osteosarcomas at a higher frequency and to a greater extent than single transgenics, indicating that c-Fos co-operates with c-Jun in osteoblast transformation and tumour formation (Wang et al., 1995). Histological analysis showed that the tumours developing in double transgenic mice contained greater amounts of neoplastic bone with fewer chondrocytes. Also, cell lines isolated from Fos-Jun double transgenic tumours expressed high levels of both transgenes and osteoblastic marker genes, further proving that osteoblasts are the target cells for c-Fos induced transformation. The function of c-Fos in osteosarcoma formation was further investigated by crossing *c-fos* transgenic mice with heterozygous and homozygous *c-fos* knockouts. Interestingly, the frequency of osteosarcoma formation and the time of onset were significantly reduced in *c-fos*+/- mice, and tumour formation was almost absent in *c-fos*-/- mice expressing

*c-fos* transgene (Wang et al., 1995), suggesting that a critical level of c-Fos is necessary for osteosarcoma development.

The molecular mechanisms by which overexpression of *c-fos* induces osteosarcomas in transgenic mice is not fully understood. Fibroblastic cell lines with a mutant allele of *c-jun* (*jun*AA) infected with *v-fos*-expressing retrovirus showed reduced tumorigenicity in nude mice. In addition, c-Fos-induced osteosarcoma was suppressed in *junAA* mice, which are c-Jun mutant mice where the two Jun N-terminal kinase (JNK) phosphorylation sites have been mutated, resulting in a mutant "non-phosphorylatable" c-Jun protein (Behrens et al., 2000), suggesting that c-Fos-induced tumorigenesis is mediated by c-Jun N-terminal phosphorylation. Our laboratory reported that overexpression of c-Fos induces osteoblast transformation, possibly by modifying the expression of cells cycle regulators (Sunters et al., 1998). Recently, *c-fos* induced growth factor/vascular endothelial growth factor D (*Figf/Vegf-D*), has been identified as a downstream growth and morphogenic effector of *c-fos*. Overexpression of *Figf/Vegf-D* in fibroblasts caused morphological alterations (Orlandini et al., 1996). Furthermore, *Figf/Vegf-D* induced angiogenesis both *in vivo* and *in vitro*, and also induced proliferation and chemotaxis in Kaposi's sarcoma immortalized cell line (Marconcini et al., 1999).

### 1.7.2.2 Chimaeric mice overexpressing *c-fos* develop chondrosarcomas

As exogenous *c-fos* is not expressed during embryogenesis in Fos-transgenic mice, in order to further identify the early target cells for c-Fos transformation, chimaeric mice overexpressing *c-fos* were generated by introducing a *c-fos* transgene into pluripotential embryonic stem (ES) cells. c-Fos-ES cell chimaeras developed chondrosarcomas with high efficiency at all skeletal sites containing cartilage at 3-4 weeks after birth (Grigoriadis et al., 1993; Wang et al., 1991; Wang et al., 1993). *In situ* hybridisation and immunocytochemistry showed high levels of exogenous *c-fos* expression in chondrogenic cells in chondrosarcomas. Cell lines isolated from chondrosarcomas expressed high levels of *c-fos*, *c-jun* and cartilage specific genes. Injection of these cells into syngeneic and nude mice induced cartilage tumours (Wang et al., 1993). These results suggest that ectopic *c-fos* transgene expression in chimaeric mice occurred at an earlier developmental stage, which may affect the common mesenchymal progenitors of chondrocytes and osteoblasts. Our laboratory has further shown

that c-Fos affects cartilage differentiation, as overexpression of *c-fos* in a chondrocyte cell line inhibited chondrocyte differentiation *in vitro*. The expression of chondrocyte marker genes was downregulated by exogenous *c-fos*, while *fra-1* and *c-jun* expression was upregulated (Thomas et al., 2000).

#### 1.7.2.3 Mice lacking *c-fos* develop osteopetrosis

Deletion of *c-fos* in mice results in the development of osteopetrosis (Wang et al., 1992) (See also section 1.7.3.1), with poorly developed cortical bone and disorganised growth plates. Although, osteoblasts in *c-fos* -/- mice express lower level of bone sialoprotein and osteocalcin (Demiralp et al., 2002), the fact that osteoblasts from mutant mice can fully support osteoclast formation *in vitro* suggests that the defect of osteogenesis may be secondary to the bone remodelling defect caused by the lack of osteoclasts (Grigoriadis et al., 1994). Recently, Demiralp et al. reported that after PTH administration, bone mineral density and trabecular bone volume were decreased in *c-fos* knockout mice, but were increased in the wild-type littermates (Demiralp et al., 2002), suggesting that deletion of *c-fos* in osteoblastic cells caused an altered responsiveness to growth factors during endochondral ossification.

### 1.7.3 Effects of *c-fos* on the haematopoietic lineage

#### 1.7.3.1 Inactivation of *c-fos*

The indispensable role of c-Fos in osteoclastogenesis is unequivocally demonstrated by the osteopetrotic phenotype in mice lacking *c-fos* gene. The *c-fos* knockout mice were generated by disrupting the *c-fos* gene in embryonic stem cells. A neomycin-resistance gene (*neo*) was fused in-frame to *c-fos* replacing part of the second exon and intron (*Bgl*II fragment), thereby deleting 483 bp of *c-fos*. Mice lacking *c-fos* are viable but are growth–retarded (Wang et al., 1992), and develop osteopetrosis due to a complete absence of functional multinucleated osteoclasts and their immediate precursors (Grigoriadis et al., 1994), the failure of tooth eruption and altered haematopoiesis are secondary to the osteopetrosis (Okada et al., 1994; Wang et al., 1992).

The defect of osteoclastogenesis in *c-fos* knockout mice is intrinsic in the osteoclast lineage, and is not caused by an altered bone marrow environment. Transplantation of mutant embryonic limbs into the wild-type kidney supported normal bone development, whereas wild-type limbs transplanted under mutant kidney cap-

sules lacked a bone marrow space and TRAP positive cells (Grigoriadis et al., 1994). Moreover, wild-type bone marrow transplantation rescued lethally irradiated newborn mutant mice, as well as bone marrow space formation and incisor eruption. In contrast, injection of mutant spleen haematopoietic stem cells into irradiated newborn wild type mice caused osteopetrosis (Grigoriadis et al., 1994). Osteoblasts isolated from wild-type and mutant calvariae supported osteoclast formation in coculture with wild-type spleen cells, while mutant spleen cells could not form osteoclasts when cultured with wild-type osteoblasts. Finally, mutant spleen cells infected with a *c-fos*-expressing retrovirus formed functional osteoclasts *in vitro* (Grigoriadis et al., 1994). Taken together, these experiments demonstrated unequivocally that the defect in *c-fos* knockout mice was cell autonomous in the osteoclast lineage. This was also shown subsequently by Udagawa and coworkers, who showed that in a mouse bone marrow and osteoblast coculture system, *c-fos* antisense oligomers only inhibited osteoclast differentiation when the oligonucleotides were added during the proliferation phase (day 0 to 4), but not during the second differentiation phase (days 4 to 6), suggesting c-Fos might affect the proliferation phase of osteoclast development (Udagawa et al., 1996).

Although osteoclast differentiation is blocked in the mutant mice, the number of F4/80 and Mac-2 positive macrophages in *c-fos* knockout bone marrow is increased, suggesting c-Fos has a divergent effect on osteoclast and macrophage lineage (Grigoriadis et al., 1994). However, there was no difference in the number of cells expressing ER-MP58, a marker for M-CSF-responsive macrophage progenitors. In addition, no differences were observed in the number of F4/80 positive macrophages in the liver, suggesting that the effects on the potential of macrophage/osteoclast precursors may be specific to the bone marrow compartment. It was also reported that c-Fos is involved in the lineage determination of osteoclasts and dendritic cells. RANKL-induced osteoclast differentiation was inhibited and dendritic cell differentiation was reciprocally stimulated by GM-CSF through suppression of *c-fos* in bone marrow cultures (Miyamoto et al., 2001). On the other hand, GM-CSF-induced dendritic cell development was inhibited by overexpression of *c-fos* (Miyamoto et al., 2001). Taken together, these data suggest that c-Fos plays a critical role in the differentiation of osteoclast precursors at the branch point where the osteoclast lineage diverges from the macrophages and dendritic cells.

The genes upstream of *c-fos* in regulating osteoclast differentiation are not clear. It was reported that *c-fos* gene can be transcriptionally activated in mononuclear phagocytes by M-CSF (Muller et al 1985; Bravo 1987), but in the absence of M-CSF, neither a *c-fos* nor a *fra-1* expressing retrovirus supported survival or proliferation of osteoclast precursors (Owens et al., 1999). Recently, a link between RANK signalling and the AP-1 expression during osteoclastogenesis has been identified (see also below).

To demonstrate the possible molecular mechanism by which c-Fos exerts its function in osteoclastogenesis, gene rescue experiments have been done both *in vivo* and *in vitro*. It was demonstrated that the N-terminal portion and the core region of Fos proteins were sufficient to rescue osteoclast differentiation. Interestingly, all Fos proteins (c-Fos, Fra-1, Fra-2, and Fos-B), but none of the Jun proteins (c-Jun, Jun-B, and Jun-D) rescued the defect of osteoclast differentiation from *c-fos*-/- spleen cells. Fra-1 has the highest rescue potential of all Fos proteins, in addition, *fra-1* transgene rescue the osteopetrosis *in vivo* (see also below) (Matsuo et al., 2000).

### 1.7.3.2 Overexpression of *c-fos*

Prolonged expression of *c-fos* enhances osteoclast differentiation *in vitro*. Bone marrow precursors infected with *c-fos*-expressing retrovirus (pMX-*c-fos*-IRES-EGFP) differentiated into TRAP positive cells even in the absence of RANKL, though the efficiency was very low (Takayanagi et al., 2002a). Overexpression of *c-fos* in integrin $\beta_3$-/- bone marrow precursors rescued the defect in osteoclastogenesis, but not the bone resorption (Faccio et al., 2003). Transfection of *c-fos* cDNA into avian osteoclast precursors induced a twofold increase in TRAP activity and osteoclastic bone resorption (Miyauchi et al., 1994). Finally, osteoclasts from Paget's patients expressed high levels of *c-fos* (Hoyland and Sharpe, 1994), which may play an important role in the increased bone remodelling of these patients.

Experiments from our laboratory have also implicated that osteoclasts are involved in c-Fos-induced tumorigenesis. Transgenic mice overexpressing deregulated *c-fos* in osteoclasts using a TRAP promoter develop large bone lesions and tumours, which contained a marked increase in the number of osteoclasts (Beedles et al., 1999). Osteosarcomas developing in Fos-Jun double transgenic mice contained a greater number of TRAP positive multinucleated osteoclast-like cells compared with c-Fos

transgenic mice, and were more remodelled (Wang et al., 1995), suggesting osteoclasts may play an important role in bone tumour remodelling and progression.

### 1.7.4 Roles of Fra-1 in bone biology

Fra-1 (Fos related antigen- 1) is one of the Fos proteins, encoded by the Fos-like–1 gene, *Fosl1*. *Fosl1* is a transcriptional target of *c-fos* in osteoclast progenitors, and induction of *Fosl1* upon growth factor stimulation is delayed compared with *c-fos* (Bergers et al., 1995; Matsuo et al., 2000; Schreiber et al., 1997). RANKL induces *fra-1* expression in a *c-fos*-dependent manner (Matsuo et al., 2000). Structurally, *c-fos* and *fra-1* show high homology in their leucine zipper and DNA-binding domains. The major difference between these two genes is that *fra-1* lacks transcriptional activation domains required for cellular transformation or transactivation and fails to activate transcription when fused to the DNA-binding domain of Gal4, while *c-fos* is a potent activator of transcription (Cohen et al., 1989). Fra-1 is expressed in extraembryonic tissues during mouse development and in brain, skin, and testes of adult mice (Schreiber et al., 1997; Schreiber et al., 2000).

#### 1.7.4.1 Overexpression of *fra-1*

Fra-1 transgenic mice were generated using H2-*fra-1*LTR construct. These mice developed osteosclerosis due to increased bone formation (Jochum et al., 2000), but no bone tumour formation occurred. Osteoblast differentiation but not proliferation was enhanced by overexpression of *fra-1*. The number of osteoclasts and resorption pits induced from Fra-1 transgenic bone marrow precursors was significantly increased than that from the wild-type (Matsuo et al., 2000). Fra-1 is more potent in inducing osteoclast formation than c-Fos *in vitro*, since infection of bipotential osteoclast/macrophage precursor cell lines with a retrovirus expressing *fra-1* but not *c-fos* dramatically stimulated the formation of osteoclast in cocultures (Owens et al., 1999). Also, as described above, overexpression of *fra-1* by retrovirus infection in spleen cells lacking *c-fos* rescued the differentiation *in vitro* more efficiently than other Fos members (Matsuo et al., 2000). The osteopetrosis in *c-fos-/-* mice was partially rescued by crossing with *fra-1* transgenic mice. With advancing age, the mice developed increased bone density similar to *fra-1* transgene-induced osteosclerosis. To further investigate whether Fra-1 can substitute for c-Fos, knock-in mice expressing *fra-1* in place of *c-fos* were generated. Fra-1 knock–in mice studies showed

that Fra-1 can fully compensate for the lack of c-Fos in bone development, but the rescue was gene dosage dependent (Fleischmann et al., 2000).

### 1.7.4.2 Inactivation of *fra-1*

Targeted disruption of the *Fosl1* gene leads to early embryonic lethality due to impaired vascularisation of the placenta (Schreiber et al., 2000). However, injection of *fra-1*-/- ES cells into tetraploid wild-type blastocysts rescued the placental defect, and in these mice, *fra-1*-/- newborns survived up to two days after birth without any organ abnormalities. Bone structures were normal, and TRAP positive osteoclasts were present, suggesting that Fra-1 is not essential for osteoclast differentiation, and regulates osteoclast formation by a mechanism different from c-Fos (Matsuo et al., 2000).

## 1.8 Signalling in osteoclast development

It's well established that RANKL binds to RANK, the receptor on osteoclast progenitors, and promotes osteoclast formation, activation and survival, but the molecular mechanisms downstream of RANK are still not clear. Three different pathways have been identified so far, and Figure 1.9 shows signalling pathways involved in osteoclast differentiation (Adapted from Karsenty and Wagner, 2002).

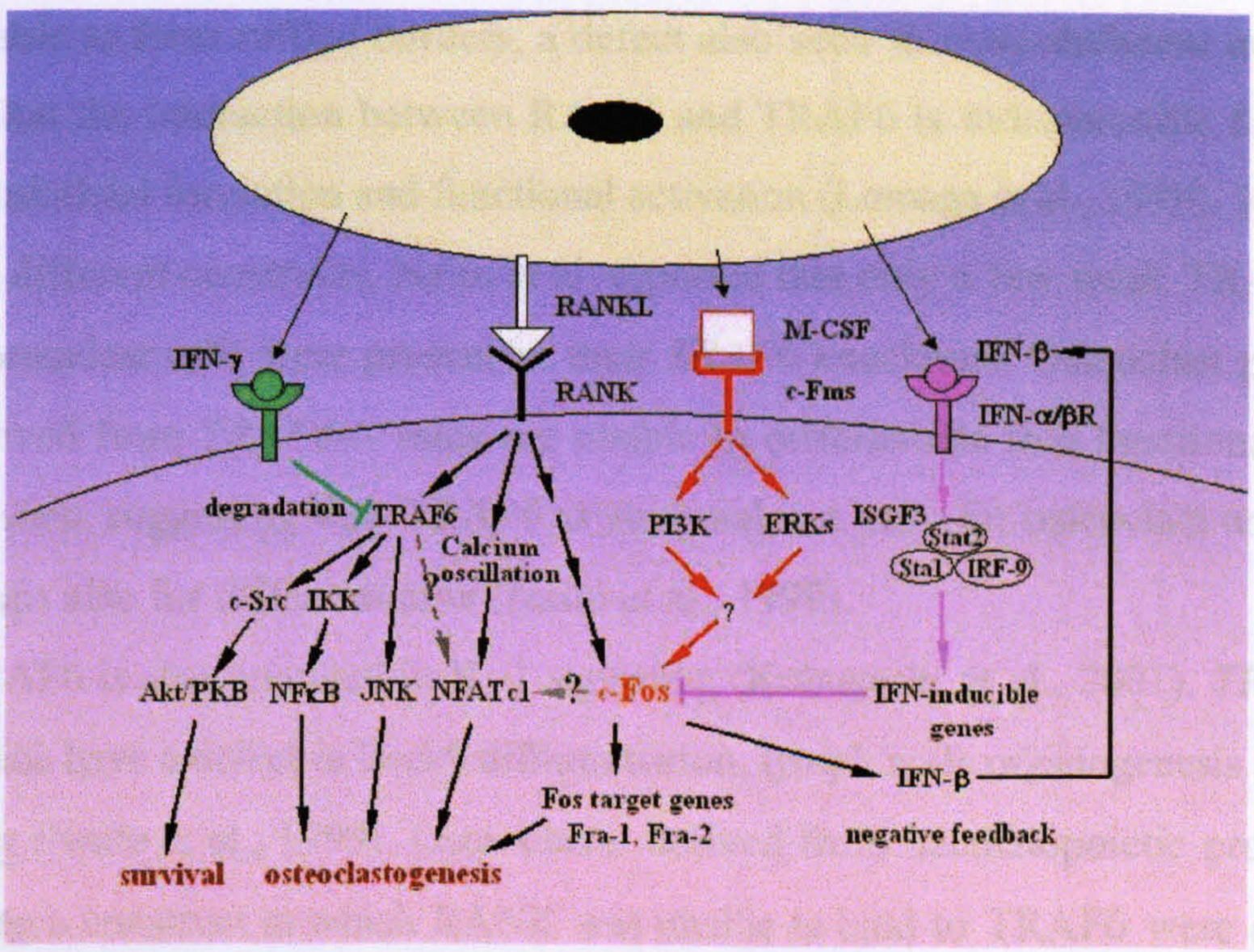



**Fig1.9 RANKL and M-CSF signalling in osteoclast differentiation**

### 1.8.1 TNF receptor-associated factors (TRAFs)

RANK's intracellular domain contains two binding sites for members of a family of proteins called TNF receptor-associated factors (TRAFs) (Darnay et al., 1999). The TRAF family proteins are cytoplasmic adapter proteins that mediate cytokine signalling. Both the TNF receptor superfamily and the Toll/interleukin-1 receptor family recruit TRAF proteins. There are six members in this family (Arch et al., 1998), among which TRAF2, TRAF5, and TRAF6 have been shown to be responsible for NF-κB activation through IκB kinase (IKK) activation by TNF receptors (Kim et al., 1999), and they have also been implicated in activation of c-Jun N-terminal kinase (JNK) (Anderson et al., 1997; Darnay et al., 1998; Wong et al., 1997c), which phosphorylates *c-jun* and activates the AP-1 complex. TRAF6, which binds to a structural motif different from the region that binds other TRAFs, is necessary and sufficient for RANK-induced NF-κB activation, while TRAF2 is responsible for the activation of JNK (Darnay et al., 1999; Wong et al., 1998). RANK activates not only NF-κB and JNK, but also the expression of c-Fos (Matsuo et al., 2000).

The role of TRAF6 involved in osteoclast differentiation is demonstrated by the osteopetrosis observed in mice lacking *TRAF6*. In *TRAF6* knockout mice, osteoclasts are present in normal numbers on bone surfaces but are non-functional, as they were unable to form ruffled borders, a defect also seen in *c-src*-deficient mice, suggesting that the interaction between RANK and TRAF6 is indispensable for osteoclast cytoskeletal formation and functional activation (Lomaga et al., 1999). However, by using different constructs, Naito et al. reported that only a few weak TRAP positive mononuclear cells were present in their *TRAF6* knockouts. Osteoclast precursor cells derived from *TRAF6*-/- mice are unable to differentiate into functional osteoclasts *in vitro*, suggesting that TRAF6 is essential not only for osteoclast activation, but perhaps also for differentiation (Naito et al., 1999).

TRAF6 is also involved in IL-1 signalling (Kobayashi et al., 2001). *TRAF6* deficient mice have a defect in B-cell differentiation, lymph node organogenesis and IL-1 signalling (Naito et al., 1999). Osteoclasts derived from haematopoietic progenitors harbouring a construct in which RANK was unable to bind to TRAF6 were dysfunctional, which can be rescued by IL-1β (Armstrong et al., 2002; Cao et al., 1996).

It was also reported that RANKL promotes osteoclast survival by activating Akt/PKB, a serine/threonine kinase that regulates cell survival by inactivating proapoptotic molecules, through a signalling complex involving c-Src and TRAF6. RANKL-induced activation of PKB and survival were significantly reduced in *c-src-/-* osteoclasts, and were blocked by adding PI3 or Src family kinase inhibitors (Wong et al., 1999a). Coexpression of TRAF6 with limiting amounts of c-Src induced a substantial increase in c-Cb1 phosphorylation, suggesting there is a cross-talk between RANKL and c-Src signalling pathway.

Deletion of other TRAF family members, such as TRAF2, 3 and 5 didn't affect osteoclast formation and function, but led to immunodeficiency with different severity (Nakano et al., 1999; Xu et al., 1996; Yeh et al., 1997), suggesting their roles in osteoclastogenesis are dispensable.

### 1.8.2 Nuclear Factor of Activated T cells (NFAT)

*NFATc1*, also known as *NFAT2* or *NFATc*, is a member of the NFAT (nuclear factor of activated T cells) family of transcription factors. It was demonstrated recently that *NFATc1* regulates terminal differentiation of osteoclasts. *NFATc1* is downstream of RANKL as the expression of *NFATc1* in bone marrow osteoclast/macrophage precursors is strongly upregulated after RANKL stimulation. M-CSF alone or IL-1 didn't produce similar effects. Deletion of *NFATc1* is lethal and mice died at day 14.5 of gestation. *NFATc1-/-* ES cells differentiated into monocytes/macrophages in the presence of M-CSF, but failed to differentiate into osteoclasts in the presence of RANKL. On the other hand, bone marrow precursors infected with a retrovirus expressing *NFATc1* differentiated into osteoclasts even in the absence of RANKL (Takayanagi et al., 2002a).

Interestingly, the expression of *NFATc1* depends on both TRAF6 and c-Fos. The induction of *NFATc1* by RANKL treatment was abolished in *c-fos-/-* spleen cells, and is significantly decreased in *TRAF6-/-* spleen cells (Takayanagi et al., 2002a). On the contrary, the expression of *TRAF6* and *c-fos* was normal in *NFATc1*-deficient precursors. Coexpression of *NFATc1* and *c-fos* synergistically activated the TRAP promoter, while such effect was not observed in coexpression of an *NFATc1* mutant (NFATc1RL) and *c-fos*, suggesting that *NFATc1* cooperates with *c-fos* in regulating osteoclast differentiation (Takayanagi et al., 2002a). RANKL also evokes

Ca2+ oscillations that lead to calcineurin-mediated activation of *NFATc1*, and therefore triggers a sustained *NFATc1*-dependent transcriptional program during osteoclast differentiation (Takayanagi et al., 2002a).

### 1.8.3 Interferons (IFNs)

In addition to its positive effects, RANKL also regulates osteoclastogenesis through some negative mechanisms so that the osteoclastic bone resorption is well controlled. IFN-β signalling has been identified recently as one of these pathways.

Similar to INF-γ, interferon-β (IFN-β) is a negative regulator of osteoclastogenesis. Mice deficient in one of the IFN-α/β receptor components, IFNAR1 (*IFNAR1*-/-), developed osteoporosis with increased number of osteoclasts (Takayanagi et al., 2002b). The fact that RANKL stimulation induced *IFN-β*, but not *IFN-α*, expression in osteoclast precursors, suggests that IFN-β is the key factor for the regulation of osteoclastogenesis. Similarly, mice lacking *IFN-β* developed osteopenia with enhanced osteoclastogenesis. The enhanced osteoclastogenesis in *IFNAR1*-/- is intrinsic to the osteoclast lineage. Haematopoietic precursors from *IFNAR1*-/- mice exhibited increased osteoclast formation in response to RANKL (Takayanagi et al., 2002b). It was shown that IFN-β inhibits RANKL-induced osteoclast formation through ISGF3 (a heterotrimeric complex consisting of Stat1, Stat2 and interferon regulatory factor9 (IRF-9))-mediated gene induction pathway (Takayanagi et al., 2002b).

Surprisingly, it has been identified that there is a signalling cross-talk between the RANKL and IFN-β systems. RANKL stimulation in bone marrow precursors induces *c-fos* expression, which binds to DNA and activates the expression of target genes including IFN-β. IFN-β then binds to its receptor on osteoclast precursors and inhibits osteoclast differentiation by suppressing the synthesis of c-Fos protein. Indeed, the inhibitory effect of IFN-β in osteoclast formation could be abrogated by overexpression of c-Fos. The fact that RANKL induced *c-fos* expression induces its own inhibitor provides an important negative feedback in regulating osteoclast formation (Takayanagi et al., 2002b).

### 1.8.4 c-Fms

M-CSF exerts its function by binding its receptor, c-Fms, on osteoclast precursors and mature osteoclasts. Recent studies showed that PI 3-kinase and ERK participate in M-CSF regulated osteoclast proliferation, differentiation and survival.

Phosphatidylinositol 3-kinase (PI3K) is a heterodimer consisting of a regulatory (p85) and catalytic (p110) subunit. It is involved in various cellular functions, including mitogenesis, survival, motility and differentiation. PI3K stimulates actin filament formation and regulates osteoclast attachment, spreading and chemotaxis in osteoclasts (Grey et al., 2000; Lakkakorpi et al., 1997). Inhibitors of PI3K inhibited osteoclastic bone resorption *in vitro* (Hall et al., 1995a; Nakamura et al., 1995). The fact that the activity of PI3K is upregulated after M-CSF stimulation, suggests that PI3K is an essential signalling effector molecule for *c-fms* (Pilkington et al., 1998).

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that transmit signals from extracellular stimuli to multiple substrates involved in cells growth, differentiation and apoptosis. There are three MAPK subfamilies: extracellular signal-regulated kinases (ERKs), JNKs and p38 kinases. The ERK family of MAPKs is essential for the survival of osteoclasts (Miyazaki et al., 2000). Inhibiting ERK activity rapidly induced osteoclast apoptosis *in vitro*, while activation of ERK remarkably prolonged survival by preventing spontaneous apoptosis. M-CSF stimulates ERK activity (Miyazaki et al., 2000) in osteoclasts. Prolonged ERK activation leads to enhanced c-Fos expression via phosphorylation-mediated stabilisation of the protein (Murphy et al., 2002), suggesting that *c-fms* mediates osteoclast differentiation and survival via the ERK/c-Fos pathway.

## 1.9 AIMS

The differentiation and function of osteoclasts are intricately controlled by a variety of genes at different stages, and closely regulated by many hormones and cytokines both systemically and locally. Previous studies showed that *c-fos* knock-out mice develop osteopetrosis due to a complete block in osteoclast differentiation. Moreover, these mice have a higher number of bone marrow macrophages, suggesting that c-Fos is indispensable for osteoclast development and acts at the branch point where macrophage and osteoclast differentiation diverge. But it is not clear how c-Fos is involved in the regulation of osteoclast development. Determining where the block

occurs in the osteoclast lineage is an important first step in understanding the function of c-Fos in the differentiation of this lineage. Thus, the first aim of this thesis is to map the expression of osteoclast- and macrophage- specific genes *in situ*, in both wild-type and *c-fos* knockout bones.

Spleen cells are widely used as a source of haematopoietic precursors in *in vitro* culture systems to analyse the mechanisms involved in osteoclast differentiation, especially for studies using osteopetrotic animal models as it is difficult to isolate bone marrow precursors from those animals. However, it is likely that spleen cells are different from bone marrow precursors, as these cells reside in a local microenvironment which is different from that of bone marrow cells, and which may be critical for osteoclastogenesis. Thus, in the second aim of this thesis, I will test the hypothesis that the haematopoietic precursors in *c-fos* knockout long bones, despite being reduced in number, have a different osteoclastogenic potential than precursors present in *c-fos* knockout spleens. This will be done by establishing M-CSF-dependent bone marrow culture from dissociated osteopetrotic long bones and assessing osteoclast differentiation and signalling in comparison to spleen populations.

Previous studies showed that numerous osteoclasts were formed in tumour tissues from c-Fos transgenic mice, and that, osteoclasts played an important role in tumour progression and tumour remodelling. The hypothesis to be tested in the third aim is that overexpression of *c-fos in vivo* perturbs osteoclast differentiation. This will be investigated by mapping the expression of osteoclast marker genes and osteoclastogenic cytokine genes *in situ* in c-Fos-induced osteosarcomas, and by analysing the consequences of *c-fos* overexpression on osteoclast differentiation and signalling from bone marrow precursors *in vitro*.

# Chapter 2

# Materials and Methods

## 2.1 General abbreviations

| | |
|---|---|
| α-MEM | α-minimal essential medium + ribonucleosides and deoxyribonucleosides |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| bp | base pairs |
| BMP | Bone morphogenetic protein |
| BSA | Bovine serum albumin |
| cDNA | complementary DNA |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| DEPC | Diethyl pyrocarbonate |
| 1,25-$(OH)_2D_3$ | 1,α25-Dihydroxy Vitamin $D_3$ |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra-acetic acid |
| EtBr | Ethidium bromide |
| FCS | Feotal Calf Serum |
| GAPDH | Glyceraldehyde Phosphate Dehydrogenase |
| $dH_2O$ | Deionised water |
| HCl | Hydrochloric acid |
| hr | hour/s |
| kb | kilobases |
| LiCl | Lithium chloride |
| min | minute/s |
| mRNA | messenger RNA |
| NaAc | Sodium acetate |
| NaCl | Sodium chloride |
| NTP | nucleotide triphosphate |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PFA | Paraformaldehyde |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RNasin | ribonuclease inhibitor |
| SDS | Sodium dodecyl sulphate |
| sec | seconds |
| SSC | standard saline citrate |
| TAE | Tris acetate-EDTA buffer |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline with Tween |
| TE | Tris-EDTA buffer |
| TESPA | 3-aminopropyltriethoxysilane |
| TRAP | Tartrate Resistant Acid Phosphatase |
| Tris | Tris(hydroxymethyl)aminomethane |
| tRNA | transfer ribonucleic acid |
| UTP | uridine triphosphate |

## 2.2 Characterisation of mutant mice and histology

### 2.2.1 c-Fos transgenic and knockout mice

Heterozygous c-Fos mutant mice (c-Fos+/-) were intercrossed, and the resulting c-Fos-/- and their control littermates were used for this project. c-Fos transgenic mice (H2-*c-fos*-LTR) were generated previously, where the murine genomic *c-fos* gene was fused to the MHC class I H2-Kb promoter (Grigoriadis et al., 1993). In addition, the 3'–untranslated region including the polyadenylation site of *c-fos* was replaced with the 3' LTR of the FBJ-MSV to ensure stability of the mRNA. Homozygous H2-*c-fos*-LTR mice develop osteosarcomas as early as 4 weeks after birth.

### 2.2.2 Genotyping

#### 2.2.2.1 Genomic DNA isolation

Tails (0.5-1cm long) were cut from c-Fos knockout and transgenic litters, and digested with 35µl 10mg/ml proteinase K in the 750µl tail buffer (50mM Tris·HCl, pH8.0; 100mM EDTA, 100mM NaCl, 1% SDS) at 55°C overnight. Samples were

mixed on Eppendorf Thermomix shaker for 5min. After adding 250µl saturated NaCl, samples were spun for 10min at 13,000rpm. 750µl of supernatant was collected, and DNA was precipitated by adding 500µl of isopropanol. The mixture was spun at 13,000rpm for 2min. After washing with 70% ethanol, the DNA pellet was dissolved in 200µl TE at 37°C for 2hr.

### 2.2.2.2 PCR genotyping *c-fos* knockout mice

Each PCR reaction mixture contained 1µl DNA template, 15pM of TonFos1, TonFos2 and TonNeo3 primers, 15mM $MgCl_2$, 32.5µM dNTPs, PCR buffer and 0.5µl Taq polymerase in 20µl reaction volume. The amplification was performed using 1min denaturation at 94°C, 1min annealing at 56°C, and 1min extension at 72°C, for 30 cycles. The PCR product was run on a 1.5% agarose gel with 0.5µg/ml EtBr. The expected PCR product size for the wild-type c-Fos allele is 366bp, and the mutant c-Fos allele is 276bp. The primer sequences used for PCR genotyping are shown in Table 2.1.

**Table 2.1 Primers for genotyping *c-fos* knockout mice**

| Primers | Sequence |
|---|---|
| TonFos1 | 5'-agc ttt tat ctc cga tga gg-3' |
| TonFos2 | 5'-ctg aca cgg tct tca cca tt-3' |
| TonNeo3 | 5'-tct gtt gtg ccc agt cat ag-3' |

### 2.2.2.3 Southern Blotting

10µg of genomic DNA were digested with the appropriate enzyme (*Pvu* II for *c-fos* knockouts, *BamH* I for *c-fos* transgenics) overnight at 37°C. The reaction contains 50µl DNA, 6µl 10× Buffer (Buffer B for *Pvu* II, Buffer E for *BamH* I) 40U enzyme and $dH_2O$ to a total volume of 60µl. Samples were run on a 0.8% agarose gel in TAE at 80V for 30min, then 95V for 3.5hr. The gel was depurinated with 0.25N HCl for 20min, denaturated with 0.5M NaOH/1.5M NaCl for 30min, and blotted overnight. The blot was set up from bottom to the top as follows: glass plate, 4 sheets of Whatman 3MM paper, gel upside down, GeneScreen (NEN Life Science Products), 4 sheets Whatman 3MM, 3cm high paper towel, glass plate and weight.

The Whatman paper and GeneScreen were presoaked in 10×SSC (20×SSC stock: 3M NaCl, 300mM sodium citrate, pH 7.2). The membrane was then washed with 50mM NaPi (1M NaPi stock: 89g $Na_2HPO_4 \cdot 2H_2O$, 3-4ml $H_3PO_4$, pH 7.2) for 20min. After crosslinking under UV at energy 1200 for 1.5min, the membrane was baked in 80°C oven for 1hr.

A 400bp *Acc*I-*Apa*LI fragment cloned in C54/7 plasmid was used as a probe for *c-fos* knockouts, and a 800bp *Bam*HI fragment cloned in pUM1 plasmid was used as a probe for *c-fos* transgenics. Probes for hybridisation were made as follows: 25-50ng linearised DNA template, in 45µl $dH_2O$ was denatured at 100°C for 5min. Denatured DNA was added to the "Ready to Go™ DNA labelling beads" (Amersham Pharmacia) and 5µl $^{32}$P-dCTP, and incubated at 37°C for 30min. The labelled probe was then purified by spun through ProbeQuant™ G-50 Microcolumns (Amersham Pharmacia) at 3000rpm for 2min, and denatured with 16.67µl of 2N NaOH at room temperature for 5min.

The membrane was prehybridised with Church's buffer (0.5M NaPi, pH7.2, 7% SDS, 1mM EDTA, pH8.0) at 65°C for 1hr in Techne Hybridisation Oven, and hybridised with 7-8ml of probe-containing Church's buffer at 65°C overnight. After washing with 40mM NaPi/ 1% SDS at 65°C for 3×15min to remove non-specific binding, the membrane was wrapped with Saran Wrap™ and exposed to X-ray film (Kodark Scientific Imaging film, X-OMAT™ AR) at - 80°C for the appropriate time. For *c-fos* knockouts, the expected size for the wild-type *c-fos* allele is 1.8kb, and the mutant *c-fos* allele is 1.3kb. For *c-fos* transgenics, the expected size for the *c-fos* transgene is 6.9kb.

### 2.2.3 Tissue preparation

Mice were killed by cervical dislocation. Bone tissues from c-Fos knockout, and c-Fos transgenic mice, and their wild-type littermates, were dissected and fixed in fresh 4% paraformaldehyde at 4°C for 24hr, and decalcified in 0.5M EDTA at 4°C for the required time. Bone tissues were paraffin embedded in Hypercentre XP (Thermoshandon) following dehydration and vacuum infiltration in a Histocentre 2 embedding station (Shandon). Tissue blocks were stored at 4°C. 5µm tissue sections were cut from paraffin embedded tissues by using a microtome (Reichert-Jung

1140/Autocut), and mounted on TESPA-treated slides. Sections were dried at 52°C overnight and stored at 4°C.

### 2.2.4 TESPA-treated slides

Slides were dipped in 10% HCl/ 70% ethanol for 10 sec, then washed in $dH_2O$ briefly. After dehydrating through 90% and 100% ethanol, slides were baked at 200°C for 2hr. Sterilised slides were dipped in 2% TESPA (3-aminoprophyltriethoxysilane) in acetone for 30sec, and washed twice in 100% acetone for 10sec. After drying at 40-60°C, slides were stored at room temperature.

### 2.2.5 Haematoxylin and Eosin staining

Paraffin embedded sections were dewaxed twice in histoclear for 15min, and rehydrated through decreasing concentrations of ethanol. Sections were stained in Haematoxylin for 5min, and washed with tap water for 5min. After destaining in acid $H_2O$ (10 drops concentrated HCl in 200ml $dH_2O$) for a few seconds, sections were dehydrated through increasing concentrations of ethanol. Then the sections were stained in Eosin for 30sec, dehydrated in 90% and 100% ethanol, and mounted with DPX mountant (BDH).

### 2.2.6 TRAP (tartrate resistant acid phosphatase) staining on slides

Slides for TRAP staining were dewaxed and rehydrated as described above. The TRAP staining solution was composed of 1mg/ml Naphthol-AS-TR-phosphate, 100mM sodium tartrate, N,N-dimethylformamide, acetate buffer (0.2M glacial acetic acid, 0.2M NaAc) and 1mg/ml Fast Red, buffer pH5.2. The sections were incubated in the staining solution at 37 °C for between 10min to 1hr, and washed with $dH_2O$, counterstained with Haematoxylin and aquamounted (BDH).

## 2.3 *In situ* hybridisation

### 2.3.1 Amplification of plasmid DNA

#### 2.3.1.1 Transformation

20ng of plasmids containing the appropriate DNA fragment were added to 100µl of competent bacteria cells (*Escherichia coli* DH5α), and incubated on ice for

30min. The bacterial cells were heat shocked at 42°C for 90sec, and incubated on ice again for 3min. 900µl of LB liquid was added to the bacteria, and incubated at 37°C for 1hr. 100µl transformed bacteria were plated out on LB Agar/Ampicillin (100µg/ml) and incubated at 37°C overnight. Several single colonies were picked from the plate and inoculated a starter culture of 3ml LB medium/Ampicillin (100µg/ml). Each culture was incubated at 37°C overnight with vigorous shaking (300rpm).

### 2.3.1.2 Small scale preparation of plasmid DNA

1.5ml from each culture was spun down. The pellet was resuspended in 100µl GTE (50mM glucose, 25mM Tris·Cl pH8.0, 10mM EDTA pH8.0), and incubating on ice for 5min. The bacterial cells were lysed with 200µl TENS (0.2NNaOH, 1% SDS) on ice for 5min. Protein was precipitated by adding150µl of 3M potassium / 5M acetate, and incubated on ice for 5min. The lysate was cleared by centrifugation at 13000g for 5min. The supernatant was transfered to a fresh tube, and the plasmid DNA was precipitated with 450µl isopropanol. The pellet was spun down and washed with 70% ethanol. After air-drying, the pellet was re-dissolved in 50µl TE/RNase (20µg/ml). 10µl of each plasmid were mini-digested with appropriate enzymes at 37°C for 1hr. The digestion was run on a 0.8% agarose gel to check the plasmids, and the correct plasmid was subjected to large scale preparation as described below.

### 2.3.1.3 Large scale preparation of plasmid DNA

100µl of the original culture was inoculated into 200ml LB medium/Ampicillin (100µg/ml), and incubated at 37°C overnight with vigorous shaking (300rpm). According to the QIAGEN plasmid purification Handbook, the bacterial cells were harvested by centrifugation at 6000×g for 15min at 4°C and resuspended in 10ml Buffer P1. The bacterial cells were lysed by adding 10ml Buffer P2 and incubated at room temperature for 5min. To clear the lysate, 10ml of prechilled Buffer P3 was added and incubated in the barrel of the QIAfilter cartridge at room temperature for 10min. The lysate was filtered into a previously equilibrated QIAGEN tip. The QIAGEN tip was washed twice with 25ml Buffer QC. DNA was eluted with 15ml Buffer QF and precipitated by adding 10.5ml room temperature isopropanol. After spun down at 1500g for 30min at 4°C, the DNA pellet was washed with 70% ethanol and air-

dried. The pellet was re-dissolved in a suitable volume of DEPC-$H_2O$ at 37°C for 30min.

### 2.3.1.4 Quantification of DNA

The concentration of DNA samples was measured by absorbance readings at 260nm and 280nm wavelength using Light Wave (WPA). The concentration of DNA was calculated by the following formula:

$A_{260}$×50×dilution factor=DNA conc (μl/ml)

### 2.3.1.5 Linearisation of plasmids

Plasmid DNA containing part of the known sequence of a gene of interest was digested overnight by appropriate restriction enzymes at 37°C to produce a linearised DNA template (Table 2.2), which was then cleaned by phenol/chloroform extraction or gel extraction.

**Table.2.2 List of probes**

| Probe | | Restriction enzymes | RNA polymerase | IIydrolysis time |
|---|---|---|---|---|
| ***Cbfa-1*** | S | *Xho* I | T3 | |
| | AS | *Not* I / *Xba* I | T7 | |
| ***osteocalcin*** | S | *Spe* I | T7 | |
| | AS | *Pst* I | T3 | |
| ***OPG*** | S | *Sac* II /*Nco* I | Sp6 | |
| | AS | *Spe I* | T7 | |
| ***RANKL*** | S | *Sac* II /*Nco* I | Sp6 | |
| | AS | *Spe* I | T7 | |
| ***MMP-9*** | S | *EcoR* I | Sp6 | |
| | AS | *BamH* I | T3 | |
| ***cathepsin K*** | S | *Spe* I | T7 | |
| | AS | *Nco* I | Sp6 | |
| ***RANK*** | S | *Xba* I | T3 | 25'58" |
| | AS | *Hind* III | T7 | |
| ***c-fms*** | S | *Stu* I | T7 | |
| | AS | *Pst* I | Sp6 | |
| ***pB15*** | S | *EcoR* I | T3 | 11'48" |
| | AS | *Sal* I | T7 | |
| ***Fra-1*** | S | *Xba* I | T7 | |
| | AS | *EcoR* I | T3 | |

### 2.3.1.6 Phenol-chloroform extraction

DEPC-$H_2O$ was added to the overnight digestion so that the final volume is 300μl. An equal volume (300μl) of phenol was added to the sample, and mixed well by shaking. The sample was spun at high speed for 5min, and the aqueous phase was removed to a fresh tube. After another phenol extraction, an equal volume of chloroform was added to the aqueous phase. The sample was mixed well by shaking and spun at high speed for 2min. The aqueous phase was removed to a fresh tube. DNA was precipitated at -20°C overnight with 1/10 volume of 3.0M NaAc (pH 5.2) (30μl) and 2.2 volume of 100% ethanol (726μl). DNA was pelleted and washed with 70% ethanol. After air-drying, the pellet was re-dissolved in a suitable volume of TE or DEPC- $H_2O$ at room temperature for 30min.

### 2.3.1.7 Gel extraction

A 1% (w/v) agarose gel was prepared by adding appropriate amount of agarose powder (ICN, Genetic Technology Grade Agarose) to TAE buffer (40mM Tris·base, 10mM EDTA and 0.1% acetic acid) and boiling to dissolve the agarose. The solution was cooled down to about 65°C, and EtBr (10mg/ml) was added to a final concentration of 0.5μg/ml. The agarose was poured into a gel tray and left to solidify. After it was set, the gel was put into the electrophoresis tank, and TAE buffer was poured into the tank to a level about 1mm above the gel surface. DNA samples with 6×loading dye (Promega) were loaded onto the gel along with 1Kb DNA ladder (Promega). Gels were run at 60-100V according to the size for the appropriate time. After electrophoresis the gel was examined under ultraviolet light and photograghs were taken by a Kodak video graph printer up 860CE.

According to the MinElute Handbook, DNA fragment was excised from the agarose gel, and weighed. 3 volumes of Buffer QG was added to 1 volume of gel, and incubated at 50°C for 10min to dissolve. The sample was applied to the MinElute column and spun for 1min. 500μl of Buffer QG was added to the spin column and spun for 1min. After washed with 750μl of Buffer PE, the MinElute column was dried by spun. 10μl of pre-warmed Buffer EB was added to the centre of the membrane and incubated at 37 °C for 5min. DNA was eluted by spun for 1min.

### 2.3.1.8 Quantification of linearised plasmid

1µl of purified linearised plasmid was run on a 0.7% agarose gel along with 1µg Lambda DNA/*Hin*d III Markers (Promega). The amount of DNA was evaluated approximately by comparing the intensity of bands to that of the ladder fragments under ultraviolet light.

## 2.3.2. *In vitro* transcription

### 2.3.2.1 Digoxygenin *in vitro* transcription

1-2µg of linearised DNA template were added to the transcription reaction along with 2µl 0.1MDTT, 4µl 5×Transcription Buffer, 2µl 10×Digoxygenin labelling mix (Roche), 1µl RNasin (40U/µl) and 2µl RNA polymerase necessary for the transcription reaction (i.e. T3, T7 or SP6 20U/µl, Promega) (Table 2.2) for 2hr at 37°C. The DNA template was digested with 5µl RNase-free DNase (1U/ul, Promega) for 30min at 37°C, and the reaction was stopped with 2µl 0.2M EDTA. The probe was precipitated overnight with 2µl 5M LiCl and 75µl 100% ethanol at -20°C. The RNA probe was pelleted by spun at 13,000rpm 4°C for 20min. The pellet was washed with 500µl 70% ethanol. After spinning for 10min at 13,000rpm at 4°C, the pellet was resuspended in 100µl DEPC-$H_2O$ and 1µl RNasin and dissolved at 37°C for 20min. 3µl RNA probe was run on a 0.8% agarose gel to check the quality and quantity of the probe. RNA probes were stored at -20°C.

### 2.3.2.2 Radioactive *in vitro* transcription

2-3µg of the DNA template were added to the transcription reaction with 0.5µl 1M DTT, 1.2µl 10mM GTP, 1.2µl 10mM CTP, 1.2µl 10mM ATP, 1µl 50µmM UTP (Promega) and 7µl $^{35}$S-labelled UTP (1100Ci/mmol), 0.5µl RNasin and the appropriate RNA polymerase. This was incubated at 37°C for 40min, fresh enzyme was added and incubated for a further hour. The template was digested with 0.5µl RNase-free DNase, 1µl RNasin, 0.5µl 1M DTT and 1µl 10mg/ml tRNA at 37 °C for 10min. The RNA probe was precipitated by the addition of 4µl 1M DTT, 4µl 5M NaCl, 20µl 3M NaAc and 400µl 100% ethanol and 160µl DEPC-$H_2O$ at -20°C overnight. The RNA probe was pelleted at 13,000 rpm for 10min, and washed twice

with 500 µl 10mM DTT in 70% ethanol. After air-drying, the pellet was resuspended in 50µl 50mM DTT in DEPC-$H_2O$. RNA probes were stored at -20°C.

The counts of probes were measured. 1 µl probe was added to 2ml of scintillation fluid in a plastic scintillation vial. The activity of the probe was measured on a liquid scintillation counter (EG&G Berthold).

### 2.3.2.3 Hydrolysis of riboprobes

If the probe was longer than 300bp, it was hydrolysed to generate smaller fragments. 50µl hydrolysis buffer containing 80mM $NaHCO_3$, 120mM $Na_2CO_3$ pH10.2 and 10mM DTT was added to the probe and incubated at 60°C for the required length of time according to each probe. The reaction was stopped by adding 50µl neutralising buffer (0.2M sodium acetate, 1% glacial acetic acid, 10mM DTT). The riboprobe was precipitated with 10mM 3M NaAc pH5.2, 1µl tRNA and 425µl 100% ethanol at -20°C overnight. The RNA probe was pelleted at 13,000rpm for 15min. After washing with 5001µl 10mM DTT in 70% ethanol, the RNA probe was resuspended in 50µl 50mM DTT. Table.2.2 shows the probes used for *in situ* hybridisation.

The expression of exogenous *c-fos* was analysed by using a transgene-specific probe, named pB15, as described previously (Grigoriadis et al., 1993). The probe is a 480bp PCR fragment derived from the FBJ-MSV, coding part of the retroviral envelope protein p15E.

### 2.3.3 *In situ* hybridisation

### 2.3.3.1 Digoxygenin-*in situ* hybridisation

Paraffin sections were dewaxed, rehydrated and postfixed with 4% paraformaldehyde. Slides were washed in PBS, and immersed in 0.1M HCl. They were then digested with proteinase K (10µg/ml in 20mM Tris·HCl, 2mM $CaCl_2$, pH7.4) at 37°C for 15 -30min. The tissues were postfixed and acetylated in 0.25% acetic anhydride in 0.1M TEA and washed in PBS.

Tissues were prehybridised using buffer containing 50% formamide, 2×SSC, 0.5×Denhardts solution and yeast tRNA, for 2hr at room temperature. Digoxygenin

labelled probes were added at 100ng/ml in prehybridisation solution, heated at 100°C for 3min and cooled on ice. Hybridisation buffer was added to the sections and overlaid with parafilm and incubated overnight at 55°C (hot start at 80°C). Slides were then washed in 5×SSC at 55°C for 5min, 0.2×SSC for 1hr, and cooled to room temperature in 0.2×SSC. Sections were then washed in 100mM Tris, 150mM NaCl pH7.5 and pretreated with 1% blocking agent for 1hr, followed by incubation with anti-DIG Fab-fragment for 1hr. Colour development was carried out in the presence of NBT (4-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-cholro-3-indolyl-phosphate) and levamisole for 5min to overnight. Slides were then washed in 10mM Tris, 1mM EDTA pH8.0, counterstained with Haematoxylin, and mounted with aquamount.

### 2.3.3.2 $^{35}$S -radioactive *in situ* hybridisation

Sections were dewaxed with xylene, rehydrated with decreasing concentrations of ethanol, and postfixed with 4% PFA/PBS for 10min. After washing with PBS, sections were digested with 10µg/ml proteinase K (37°C, 30min) in PBS and postfixed with 4%PFA/PBS for 10min. Sections then were sequentially washed with PBS, incubated in 0.2 N HCl for 10min. After washed with PBS, sections were acetylated with 0.25% acetic anhydride in the presence of 0.1M triethanolamine for 10 min, dehydrated with increasing concentrations of ethanol, and air-dried. Hybridisations with $^{35}$S-labelled complementary RNAs were performed in a humidified chamber in a solution containing 50% formamide, 10% dextran sulphate, 1×Denhardt's solution, 600mM NaCl, 10mM Tris·HCl, 1mM EDTA, 50mM DTT, 0.25% SDS, and 200µg/ml tRNA overnight at 50°C.

After hybridisation, sections were washed with 5×SSC at 50°C, with 50% formamide/2×SSC at 50°C for 30min, and then with 10mM Tris·HCl (pH7.6), 500mM NaCl, 1mM EDTA (TNE) at 37°C for 10min. Sections were treated with 10µg/ml ribonuclease A in TNE (37°C, 30min). After being washed with TNE, sections were incubated once with 2×SSC (50°C, 20min) and twice with 0.2×SSC (50°C, 20min). Sections were dehydrated with increasing concentrations of ethanol and air-dried. Slides were then placed on X-ray films (Kodak BioMax MR Film), and autoradiographs were obtained after overnight exposure at room temperature. Slides were

dipped into emulsion and stored at room temperature or 4°C (after 4 days) for the times estimated from the intensity of expression on X-ray film. After developing, sections were counterstained with Haematoxylin and Eosin and mounted with DPX mountant.

## 2.4 Immunohistochemistry

Paraffin sections were dewaxed twice in Histoclear for 15 min, and rehydrated through decreasing concentrations of ethanol. Sections were trypsinised in 1% trypsin in TBS at 37°C for 15min. To prevent non-specific background, sections were blocked with serum in TBS at room temperature for 30min, 20% normal goat serum for OPG and 20% normal rabbit serum for RANKL. Sections were incubated with primary antibodies, goat polyclonal anti-Mouse RANKL (Santa Cruz) and rabbit polyclonal anti-Human OPG (Insight Biotechnology), at room temperature for 2hr or at 4°C overnight. After washing with TBS for3×5min, sections were incubated with secondary antibodies for 1hr, biotinylated rabbit anti-goat IgG (Dako) for RANKL and goat anti-rabbit for OPG. After washed with TBS, sections were incubated with ABC (StrepABComplex/AP, Dako) for 30min. Colour was developed with Sigma Fast Red TR/Napthol AS-MX Tablet set for 2min until visible. Sections were washed in TBS, counterstained with haematoxylin for 45sec and mounted with aquamount.

For F4/80 staining, after dewaxed and rehydrated, sections were incubated in 0.42% $H_2O_2$/methanol for 40min to block the endogenous hyperoxidase, and washed twice with PBS. To prevent non-specific background, sections were blocked with 1:100 rabbit serum for 30min. Primary F4/80 antibody was added to the sections and incubated at room temperature for 90min. After washing three times in PBS, sections were incubated with secondary antibody (1:100 biotinylated rabbit anti-rat IgG) for 45min. ABC (Vectastain) was made by adding one drop A and one drop B to 2.5 ml PBS at 30min. After washing three times in PBS, sections were incubated with ABC for 45min, and washed twice with PBS. Colour was developed with DAB (Vectastain) for 2-5min until visible. Sections were counterstained with Haematoxylin and dehydrated through increasing concentrations of ethanol. After dipped in Histoclear, sections were mounted with DPX mountant.

## 2.5 Cell culture

### 2.5.1 Bone marrow cultures

Mice were killed by cervical dislocation, and the tibias and femurs were removed and dissected free from adhering soft tissues. Long bones isolated from c-Fos transgenic mice either had no tumours, or had small tumours not affecting most part of the bone marrow space. To make sure that exogenous c-Fos was expressed in transgenic bone marrow cells, RT-PCR for the c-Fos transgene was performed in each experiment, as described below in table 2.3.

The bone marrow was flushed with α-MEM (Sigma) containing 10% FCS either from Gibco BRL (Paisley, UK) or Summit (Denver, CO, USA) using a 10ml syringe attached to a 25-gauge needle. For the newborn mice and adult c-Fos knockout mice, bone tissues were chopped by using a sterilized scalpel blade. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension, and filtered through a cell strainer (70μm Nylon, BD Falcon™). The bone marrow cells were spun down at 1200rpm for 5min, resuspended in 15ml α-MEM containing 10% FCS and 25ng/ml recombinant murine M-CSF (Genetics Institute), and incubated overnight in a 75cm$^2$ flask (TPP) at 37°C. The next day, non-adherent cells were harvested and haematopoietic cells were counted. 90μl trypan blue in 2% glacial acetic acid was added to 10μl cell suspensions. Two times 10μl mixture was added into a haemocytometer, cells not stained blue were counted. The concentration of the cell suspension was calculated as follows:

$$(\text{No.1} + \text{No.2})/2 \times 10 \times 10^4 = \text{Cell conc (cells/ml)}$$

The appropriate volume of cell suspension was spun down at 1200rpm for 5min. Cells were plated into 96-well tissue culture plates (TPP) at a cell density of $5\times10^4$cell/well in 200μl α-MEM-10% FCS medium supplemented with 25ng/ml M-CSF and either 5ng/ml recombinant mouse sRANKL (AMGEN) or 30ng/ml of recombinant human sRANKL (Autogen Bioclear/Santa Cruz). Cells were fed every 3 days with 200μl fresh medium. For c-Fos transgenic bone marrow culture, cells were cultured with different concentrations of M-CSF (0, 10, 30 and 100ng/ml) and RANKL (0, 1, 5, 10, 50, 100ng/ml). Cells were fixed on day 5 to day 7 for TRAP staining, and day 8 to day 10 for analysis of resorption pits.

### 2.5.2 Spleen cultures

Spleens were removed, mashed and filtered through a cell strainer. Cells were spun down and resuspended in α-MEM containing 10% FCS, and incubated overnight in M-CSF (25ng/ml) in a 75cm$^2$ flask. The next day, non-adherent cells were harvested and spun down. Cells were plated into 96-well plates at a cell density of 5×10$^5$cells/well in 200µl α-MEM-10% FCS supplemented with M-CSF and RANKL. Cells were fed every 3 days with 200µl fresh medium.

### 2.5.3 TRAP staining

After culture for 5-7 days, cells were washed in PBS, fixed in 4% paraformaldehyde/ PBS for 15min at room temperature. After washing with 1x PBS, cells were stained for acid phosphatase at 37°C at the presence of 0.05M sodium tartrate using the Leukocyte Acid Phosphatase kit (SIGMA) for the appropriate time. TRAP positive cells containing at least three nuclei were counted.

### 2.5.4 Analysis of resorption pit formation

Ivory dentine slices cut to 5mm diameter wafers were cleaned by ultrasonication in distilled water, washed, sterilised by immersion in 100% ethanol overnight, and stored dry at room temperature. Cells were plated the same as on plastic. On day 5 to 7, concentrated HCl was added to the medium (82µl per 100ml medium). Cells were fixed on day 8 to 10, and stained for TRAP activity. After cells were removed by brushing with a toothbrush, the dentine slices were stained with 1% Toluidine Blue for 10sec. The resorption pits were displayed on the screen by using a microscope (Leica) and video camera (Sony), and the number of pits was quantified using the point-counting method. The screen was divided into 10mm×10mm squares, and the pits overlying each grid intersection were counted. The percentage of resorption was quantified as the number of counted pits divided by the whole area of the slice.

### 2.5.5 Statistics

All the experiments were repeated at least three times. Values presented are the mean±SE of triplicate cultures from a representative experiment. The differences between different groups were analysed by unpaired Students t-test.

### 2.5.6 Apoptosis assay

The apoptosis of cells was analysed by TUNEL (TdT-mediated dUTP nick end labelling) staining using the In Situ Cell Death Detection, POD kit (Roche). Cells grown on glass coverslips were fixed with PFA for 1hr at room temperature and rinsed in PBS. The cells were permeabilised (0.1% Triton X-100 in 0.1% sodium citrate) for 2min on ice, and rinsed twice in PBS for 5min. Cells were incubated with TUNEL reaction mixture (1:10 dilution of terminal transferase solution in label solution) at 37°C for 1hr. Coverslips were rinsed three times in PBS for 5min. After mounted with Vectashield (VECTOR), the apoptotic cells were observed under fluorescence microscope.

## 2.6 RNA manipulation

### 2.6.1 Total RNA isolation from cultured cells

Nonadherent cells were spun down at 1200rpm, and lysed with 300µl solution D (4M guanidinium, 25mM sodium citrate, 100mM β-mercaptoethanol, 0.5% lauroylsarcosine). Cells grown in 6-well plates were lysed with 300µl solution D and scraped with a rubber scraper and transferred to a falcon tube. After 30µl 2M sodium acetate (pH 4.0), 300µl saturated phenol and 76µl chloroform were added, samples were mixed thoroughly by vortexing for 10sec, and incubated on ice for 15min. The samples were spun at 10,000rpm for 20min at 4°C, the aqueous phase were transferred to a fresh tube and 300µl isopropanol was added and precipitated overnight at -20°C. Samples were then spun at 10,000rpm for 20min at 4°C, the supernatant was removed and the pellet was dissolved in 300µl solution D. This solution was transferred to an eppendorf tube and precipitate with 600µl ethanol overnight at -20°C. The samples were then pelleted by spun at speed 13,000rpm for 10min at 4°C. After washed with 75% ethanol and air-dried, the pellet was redissolved in 20 to 50µl of DEPC-$H_2O$ and stored at -80°C.

Total RNA was also isolated using RNeasy Mini Kit (Qiagen). Cells were lysed with 350µl Buffer RLT (10µl β-mercaptoethanol per 1ml Buffer RLT). The lysate was homogenized by spinning through a QIAshredder spin column for 2min at 13,000rpm. 350µl 70% ethanol was added to the homogenized lysate and mixed well

by pipetting. Samples were applied to an RNeasy mini column, and spun for 15sec at 13,000rpm. The flow-through was discarded. The RNeasy column was washed by adding 700μl Buffer RW1 and spun for 15sec at 13,000rpm. Contaminants were removed by adding 500μl Buffer RPE to the column and spun for 15sec at 13,000rpm. Another 500μl Buffer RPE was added, and the column was spun for 2min at 13,000rpm. To make sure there was no ethanol contamination, the RNeasy column was placed in a fresh 2ml collection tube and spun for another minute. RNA was eluted with 30-50μl RNase-free $H_2O$ and spun for 1min at 13,000rpm. RNA samples were stored at -80°C.

### 2.6.2 Isolation of total RNA from tissues

Limbs from *c-fos* knockouts and their littermates, tumour tissues from c-Fos transgenic mice, were dissected free of soft tissues and put in 8ml solution D (4M Guanidine thiocyanate, 25mM sodium citrate, 100mM β-mercaptoethanol. 0.5% lauroylsarcosine, 0.1% antifoam A). Sample was homogenized in Polytron for 45sec at speed 6. 0.8ml 2M NaAc (pH 4.0), 8.0ml phenol and 1.6ml chloroform were added to the sample, and mixed well by vortexing 20sec. After incubating on ice for 15min, each sample was spun at speed 10,000rpm for 15min at 4°C. The supernatant was transferred to a fresh tube, and RNA was precipitated by adding 7ml isopropanol at -20°C overnight. RNA pellet was spun down at speed 5000rpm for 15min at 4°C. After washed with 70% ethanol, RNA was dissolved in a suitable volume of DEPC-$H_2O$.

### 2.6.3 Quantification RNA

The concentration of RNA samples was measured by absorbance readings at 260nm and 280nm wavelength using Light Wave (WPA). The concentration of RNA was calculated by the following formula:

$A_{260}$ ×40 ×dilution factor=RNA conc (μl/ml)

### 2.6.4 Northern blotting

The gel tank, tray and combs were washed with 0.1N NaOH, and rinsed thoroughly with $dH_2O$. The gel was made of 3.6g agarose, 8.0ml 50×MOPS (1M MOPS, 0.25M NaAc, 0.05M EDTA, pH 7.0) and 325ml $H_2O$. After the mixture was boiled

and cooled down to 65°C, 67ml formaldehyde was added. For the samples, 20µg RNA in 20µl DEPC-$H_2O$ were mixed with 20µl formamide, 7µl formaldehyde, 1µl 50×MOPS and 2µl bromophenol blue, and heated at 70°C for 5min and cooled down on ice. The RNA samples were run on the gel in 1×MOPS at 100V for 30min, then 120V for 4hr with pumping. The gel was washed in 10×SSC for 20min with shaking, and blotted with Genescreen (presoaked in 10×SSC) overnight (see protocol for Southern blotting). The blot was washed in 50mM NaPi for 10min, and shaking for another 10min. After crosslinking under UV at energy 1200 for 1.5min, the blot was baked in 80°C oven for 1hr. Hybridisation was performed the same way as Southern Blotting. The blot was rehybridised with different probes after stripped in stripping buffer (2mM EDTA, 1% SDS) at 80°C for 30min.

### 2.6.5 RT-PCR (Reverse Transcription Polymerase Chain Reaction)

1µg of total RNA was used to synthesise cDNA. The RNA was heated to 70°C for 5min together with 0.5µg oligo-dT primer. After cooling down to 6°C, 5µl M-MLV RT Buffer, 1.25µl 10mM dNTPs, 1µl M-MLV RT (RNase $H^-$) (Qiagen) and 3.75µl DEPC-$H_2O$ were added to each reaction and heated to 40°C for 60min. The enzyme was inactivated by heating to 70°C for 15min. 3µl of RT product was used for PCR. Each PCR reaction mixture contained 10pM of reverse and forward primers, 3µl $MgCl_2$ (25mM), 1.25µl dNTPs (10mM), 5µl 10×PCR buffer and 0.5µl Taq polymerase (Promega) in a final volume of 50µl. The amplification was performed for indicated cycles shown in Table 2.3 using 1min denaturation at 94°C, 1min annealing at indicated temperature, and 1min extension at 72°C. PCR product was run on a 2% agarose gel with EtBr staining. Where indicated RT-PCR gels were scanned by densitometry using SigmaGel software.

**Table.2.3 List of primers**

| Primers | | Sequence 5'-3' | ATM | Length | Cycles |
|---|---|---|---|---|---|
| ***TRAP*** | ***For*** | agc agc caa gga gga cta cgt t | 56°C | 238bp | 28 |
| | ***Rev*** | tcg ttg atg tcg cac aga gg | | | |
| ***CTR*** | ***For*** | gtc ttg caa cta ctt ctg gat gc | 58°C | 255bp | 28 |
| | ***Rev*** | aag aag aag ttg acc acc aga gc | | | |
| ***RANK*** | ***For*** | aca cct gga atg aag aag ata aat g | 60°C | 448bp | 26 |
| | ***Rev*** | ctt cat ctc tgt ggt agt agt ggc t | | | |
| ***c-fms*** | ***For*** | aac aag ttc tac aaa ctg gtg aag g | 60°C | 753bp | 22 |
| | ***Rev*** | gaa gcc tgt agt cta agc atc tgt c | | | |
| ***F4/80*** | ***For*** | gaa ttc tcc ttg tat atc atc agc | 56°C | 157bp | 26 |
| | ***Rev*** | gaa tct tgg cca aga aga gac | | | |
| ***FOS-tg*** | ***For*** | acc tgg tgc tgg att gta tct agt gc | 60°C | 188bp | 28 |
| | ***Rev*** | gcg gta gtc cct gtt ccc act c | | | |
| ***fra-1*** | ***For*** | cgc agg ccc tgc gag cag atc | 60°C | 279bp | 40 |
| | ***Rev*** | gcc aga acc acc tgg gtc ctt | | | |
| ***fra-2*** | ***For*** | aag tgt cgg aac cgt cgc cgg gag | 60°C | 539bp | 40 |
| | ***Rev*** | ttc aag gag tct gat gac tgg tcc | | | |
| ***IFN-β*** | ***For*** | cca cag ccc tct cca tca act ata agc | 60°C | 372bp | 30 |
| | ***Rev*** | agc tct tca agt gga gag cag ttg agg | | | |
| ***TRAF6*** | ***For*** | gca agt atg agt gtc cca tct g | 60°C | 353bp | 35 |
| ***v*** | ***Rev*** | cct ggg aca atc ctc aat aat g | | | |
| ***GAPDH*** | ***For*** | acc aca gtc cat gcc atc ac | 60°C | 452bp | 24 |
| | ***Rev*** | tcc acc acc ctg ttg ctg ta | | | |

Abbreviation: ATM, annealing temperature; For, forward; Rev reverse.

# Chapter 3

## *In situ* expression studies in c-Fos knockout and transgenic mice

## 3.1 Introduction

Initial experiments were performed to investigate the extent to which osteoclasts and osteoblasts were affected when c-Fos levels were altered *in vivo*. Thus, the first part of my thesis was to examine the expression of osteoclast, osteoblast and macrophage marker genes *in situ*, in bones of mutant mice that either lack c-Fos, or in bone tumours of transgenic mice that overexpress c-Fos.

Although it has been known for a long time that *c-fos* knockout mice develop osteopetrosis due to a complete absence of osteoclasts, it's still not completely clear at which point osteoclast development is blocked. To investigate this, the expression of marker genes which represent different stages of osteoclast and macrophage differentiation were analysed by *in situ* hybridisation and immunohistochemistry.

## 3. 2 Expression of osteoclast and macrophage marker genes in *c-fos* knockout long bones

Histochemical staining for TRAP activity showed that there were many TRAP positive multinucleated osteoclasts and mononuclear precursors present in regions distal to the growth plate and along the periosteal and endosteal bone surfaces of wild-type mice (Fig.3.1.1 A,C). TRAP positive cells were completely absent in *c-fos* knockout long bones (Fig.3.1.1 B,D). As previously reported (Grigoriadis et al., 1994), this suggests that in the c-Fos knockouts, fully differentiated osteoclasts as well as their post-mitotic mononuclear precursors are absent. This was now confirmed by *in situ* hybridisation for Cathepsin K, which is also expressed at the same stages of osteoclast differentiation as TRAP. Strong expression of Cathepsin K was observed in multinucleated osteoclasts and mononuclear precursors in wild-type mice, but no expression was observed in knockout mice (Fig.3.1.2 A-D).

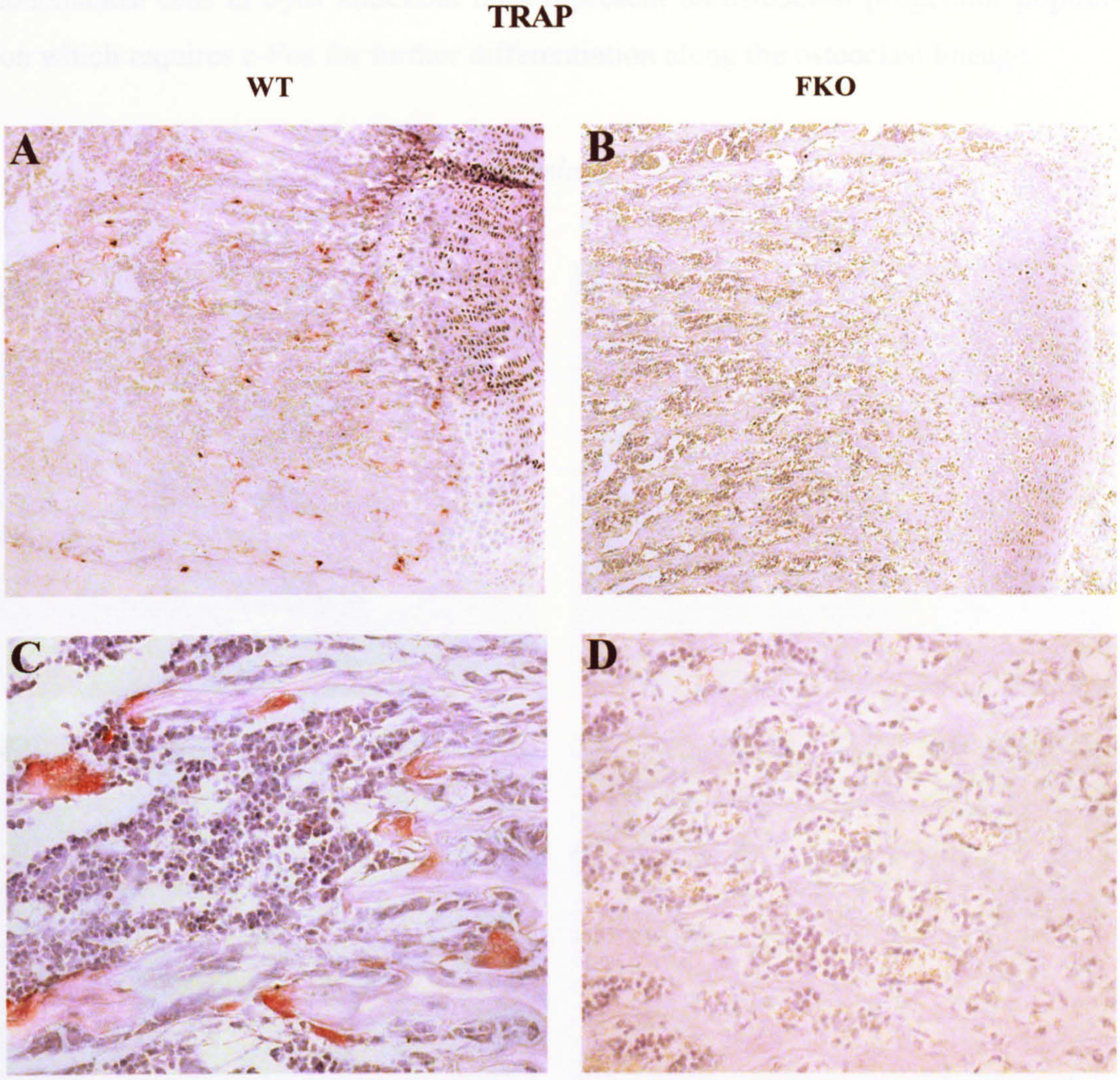



**Fig.3.1.1 TRAP-staining of long bones from wild-type and *c-fos* knockout mice.** TRAP-positive multinucleated and mononuclear cells are observed adjacent to bone surfaces in wild-type long bones (A,C), whereas *c-fos* knockout bones lack TRAP-positive cells (B,D). Original magnification: A, B ×10; C, D ×40.

*In situ* hybridisation for MMP-9, which is an early marker of the osteoclast lineage, showed strong expression in multinucleated osteoclasts and mononuclear precursors of wild-type mice, and interestingly, expression was also observed in mononuclear osteoclast precursors of the knockouts (Fig.3.1.3 A-D). Similarly, *RANK* was expressed in multinucleated osteoclasts and mononuclear precursors of wild-type mice, and was only expressed in mononuclear precursors of the knockouts (Fig.3.1.4 A-F). These observations suggest that the *MMP-9*- and *RANK*-positive

mononuclear cells in c-*fos* knockout mice represent an osteoclast progenitor population which requires c-Fos for further differentiation along the osteoclast lineage.

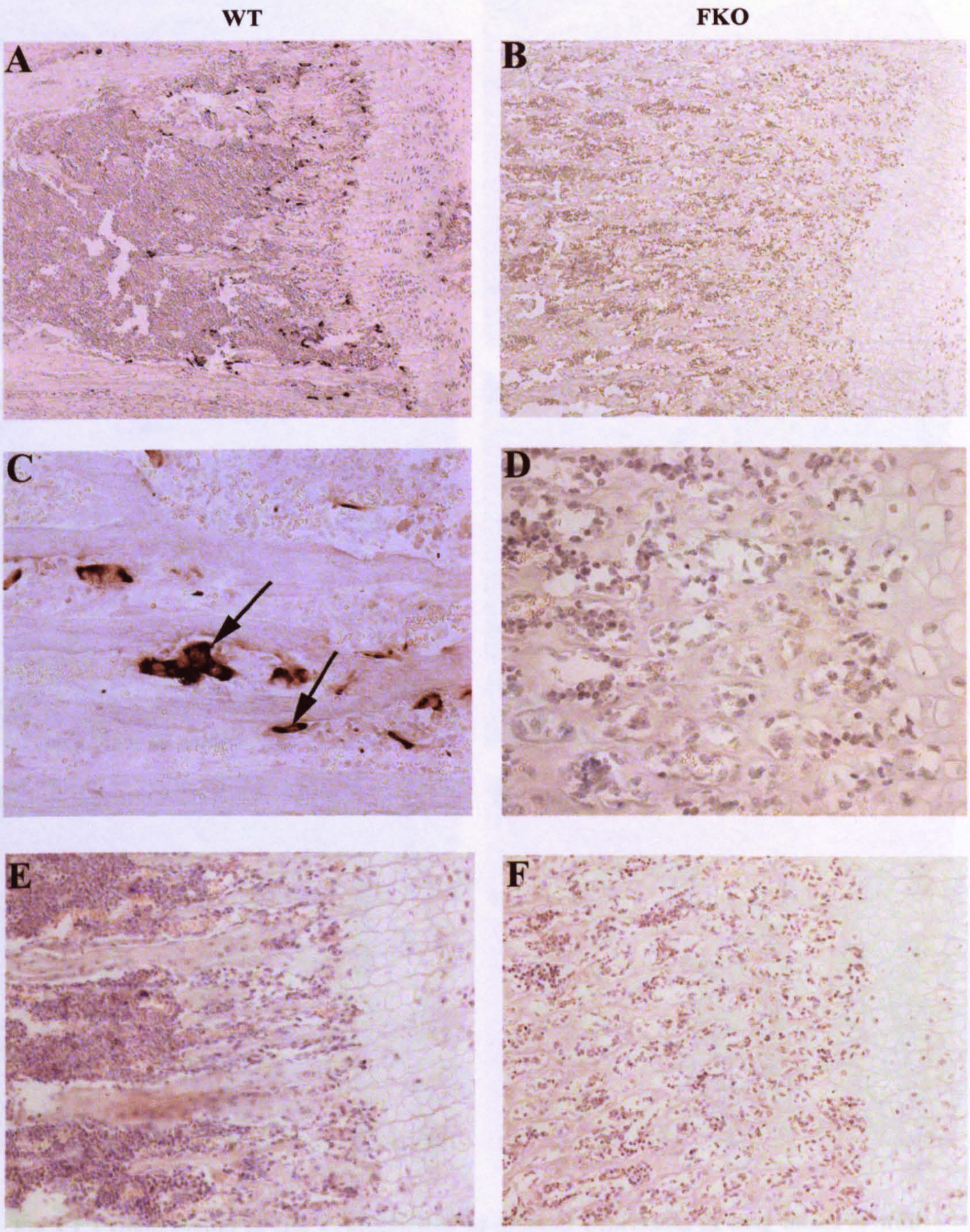



**Fig.3.1.2 Digoxygenin *in situ* hybridisation of *Cathepsin K* expression in long bones of wild-type and *c-fos* knockout mice.** *Cathepsin K* is expressed in multinucleated osteoclasts and mononuclear precursors of wild-type long bones (A,C, arrow), but is absent in *c-fos* knockout bones (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

***MMP-9***

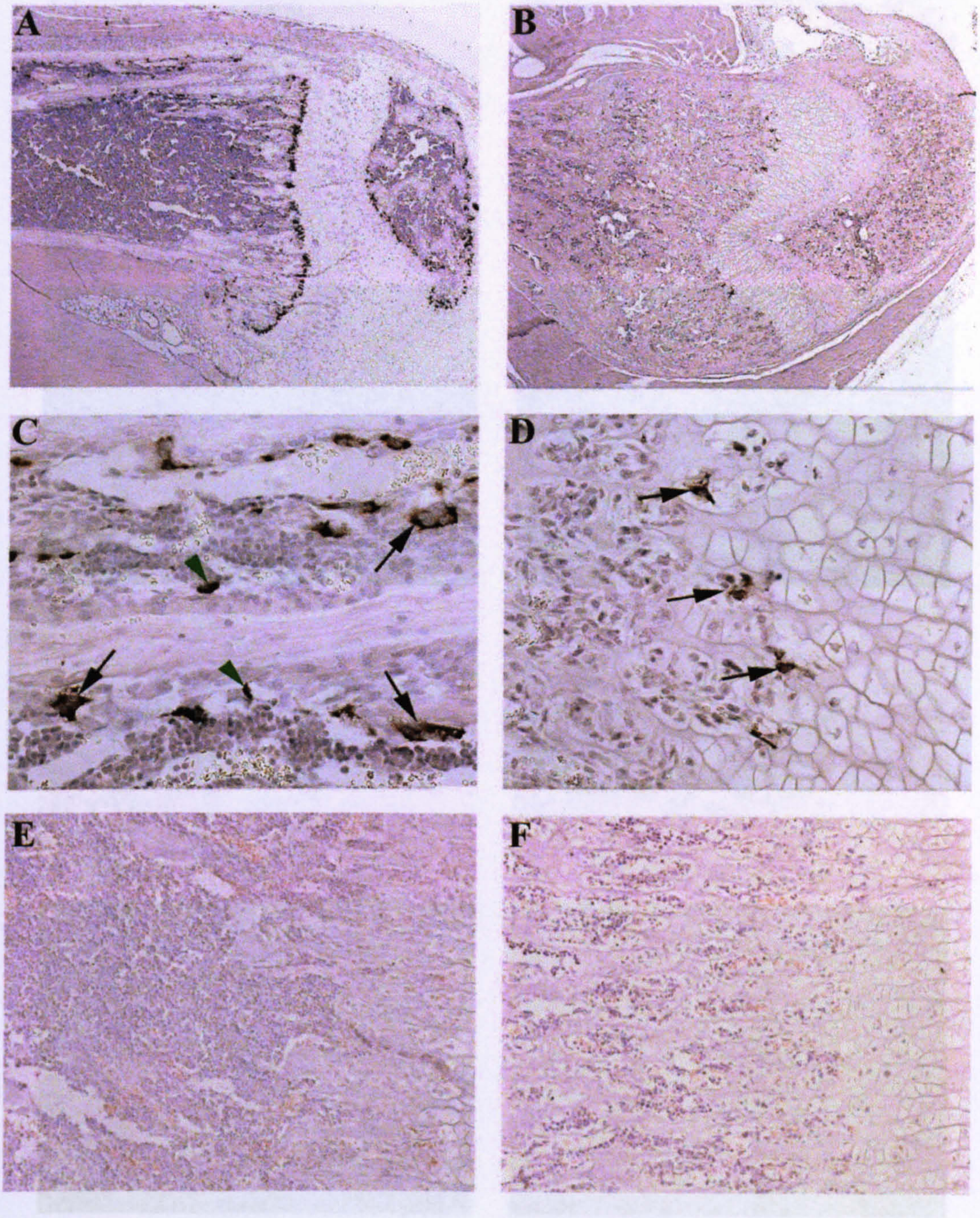



**Fig.3.1.3 Digoxygenin *in situ* hybridisation of *MMP-9* expression in long bones of wild-type and *c-fos* knockout mice.** *MMP-9* is strongly expressed in multinucleated osteoclasts (arrows) and mononuclear precursors (arrowheads) of wild-type long bones (A,C), but is only expressed in mononuclear cells (arrows) of mutant bones (B,D). E and F show sense controls. Original magnification: A, B ×5; C, D ×40; E, F ×20.

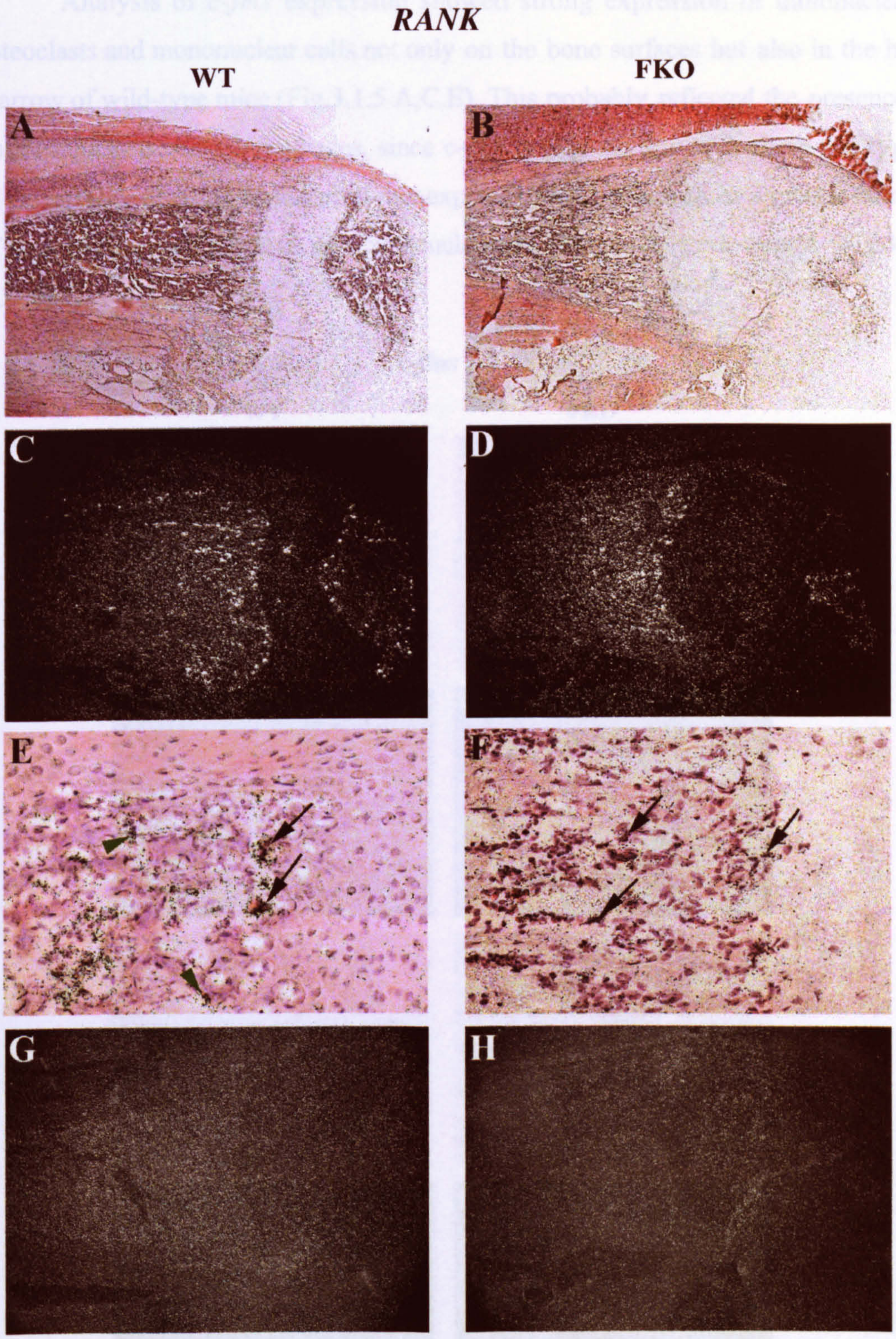



**Fig.3.1.4 Radioactive *in situ* hybridisation of *RANK* expression in long bones of wild-type and *c-fos* knockout mice.** *RANK* is strongly expressed in multinucleated osteoclasts (arrows) and mononuclear precursors (arrowheads) in wild-type bones (A,C,E). More mononuclear cells (arrow) express *RANK* in mutant bones (B,D,F). G and H show sense controls. A, B, E, F, bright field; C, D, G, H, dark field. Original magnification: A-D, G, H ×5; E, F ×40.

Analysis of *c-fms* expression showed strong expression in multinucleated osteoclasts and mononuclear cells not only on the bone surfaces but also in the bone marrow of wild-type mice (Fig.3.1.5 A,C,E). This probably reflected the presence of both osteoclasts and macrophages, since c-*fms* is expressed in both these cell types. In the absence of c-*fos*, however, c-*fms* expression was observed in a greater number of mononuclear cells but in no multinucleated cells which were absent (Fig.3.1.5 B,D,F).

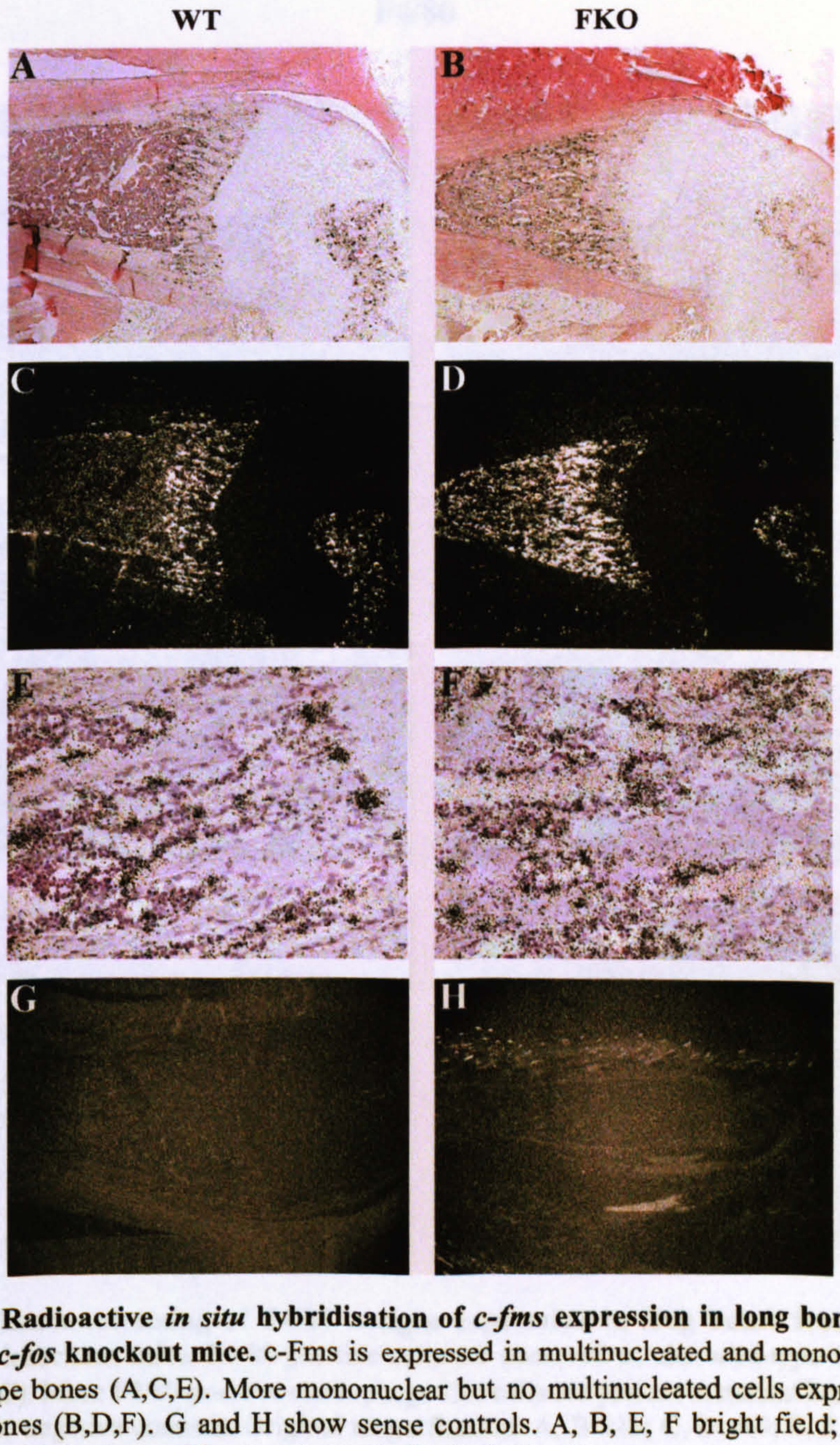



**Fig.3.1.5 Radioactive *in situ* hybridisation of *c-fms* expression in long bones of wild-type and *c-fos* knockout mice.** c-Fms is expressed in multinucleated and mononuclear cells in wild-type bones (A,C,E). More mononuclear but no multinucleated cells express *c-fms* in mutant bones (B,D,F). G and H show sense controls. A, B, E, F bright field; C, D, G, H dark field. Original magnification: A-D, G, H ×5; E, F ×40.

Since there were no osteoclasts in c-*fos* knockout mice, these results suggested that there was an increase in macrophages and this was examined further using a macrophage-specific marker. Immunostaining for F4/80 antigen showed positive staining cells in the wild-type bone marrow, while a greater number of F4/80-positive cells were observed in the knockouts (Fig.3.1.6 A-D). This confirms the increase in cells of the macrophage lineage and supports previous observations (Grigoriadis et al., 1994).

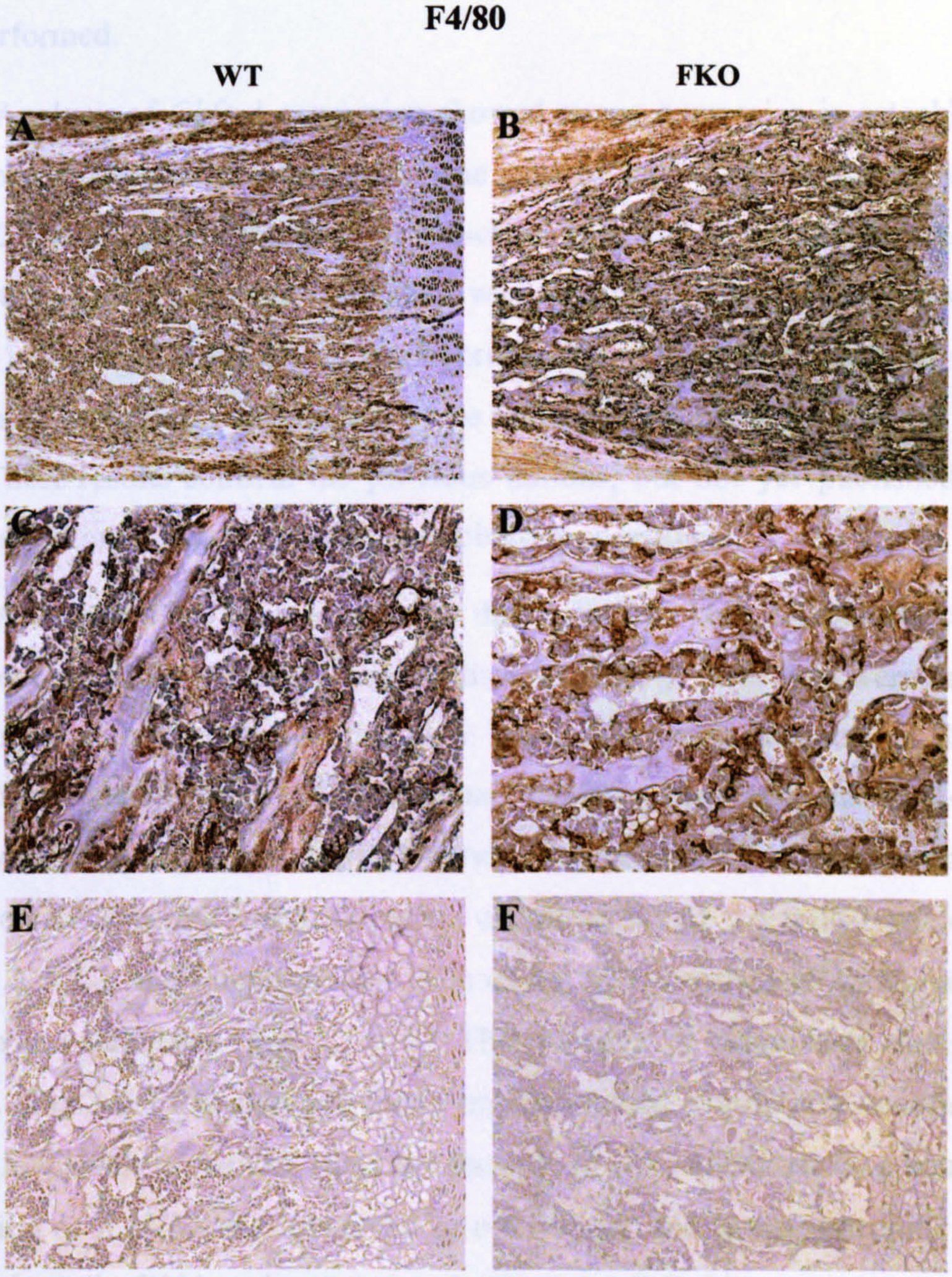



**Fig.3.1.6 Immunostaining of F4/80 antigen expression in long bones from wild-type and *c-fos* knockout mice.** F4/80-positive cells are observed in the wild-type bone marrow spaces (A,C). More F4/80-positive macrophage-like cells are present in mutant bones (B,D). E and F show negative controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

## 3. 3 Expression of osteoblast marker genes in *c-fos* knockout long bones

Previous data in *c-fos* knockouts implied that osteoblasts were normal (Grigoriadis et al., 1994). However, this was based only on their ability to support osteoclast differentiation, as the expression of marker genes had not been analysed. Thus, in order to further rule out the possibility that the defect in osteoclasts in the absence of *c-fos* is due to osteoblasts, *in situ* hybridisation and immunohistochemistry of osteoblast marker genes and genes that are critical for osteoclast differentiation were performed.

Analysis of *Cbfa-1* expression showed strong expression in osteoblasts, prehypertrophic chondrocytes and some bone marrow cells of the wild-type long bones (Fig. 3.2.1 A,C). Similarly, in *c-fos* knockout long bones, despite the altered bone architecture, strong expression of *Cbfa-1* was observed in osteoblasts and some bone marrow cells (Fig.3.2.1 B,D). *In situ* hybridisation of *osteocalcin* showed strong expression in osteoblasts of both wild-type and *c-fos* knockout long bones (Fig.3.2.2 A-D). These results confirm the previous notions, but not yet published, that osteoblasts lacking *c-fos* do not show any obvious phenotype.

Although it has been established that c-*fos* knockout osteoblasts could support osteoclast differentiation (Grigoriadis et al., 1994), these data were based on *in vitro* studies with no molecular analyses. Thus, in the next experiments, the expression of two osteoblast-derived factors that regulate osteoclastogenesis, *RANKL* and *OPG*, were analysed *in situ*. *In situ* hybridisation of *RANKL* showed strong expression in osteoblasts and prehypertrophic chondrocytes of the wild-type long bones (Fig.3.2.3 A,C), and immunolocalisation of RANKL comfirmed strong protein expression in osteoblasts (Fig. 3.2.3 E). The majority of osteoclasts didn't express *RANKL* (Fig.3.2.3 C), although some multinucleated osteoclasts occasionally expressed low levels of *RANKL* (data not shown). In *c-fos* knockout long bones, strong expression of *RANKL* was observed in osteoblasts and prehypertrophic chondrocytes, at both the RNA and protein levels (Fig.3.2.3 B,D,F,).

*Cbfa-1*

WT FKO

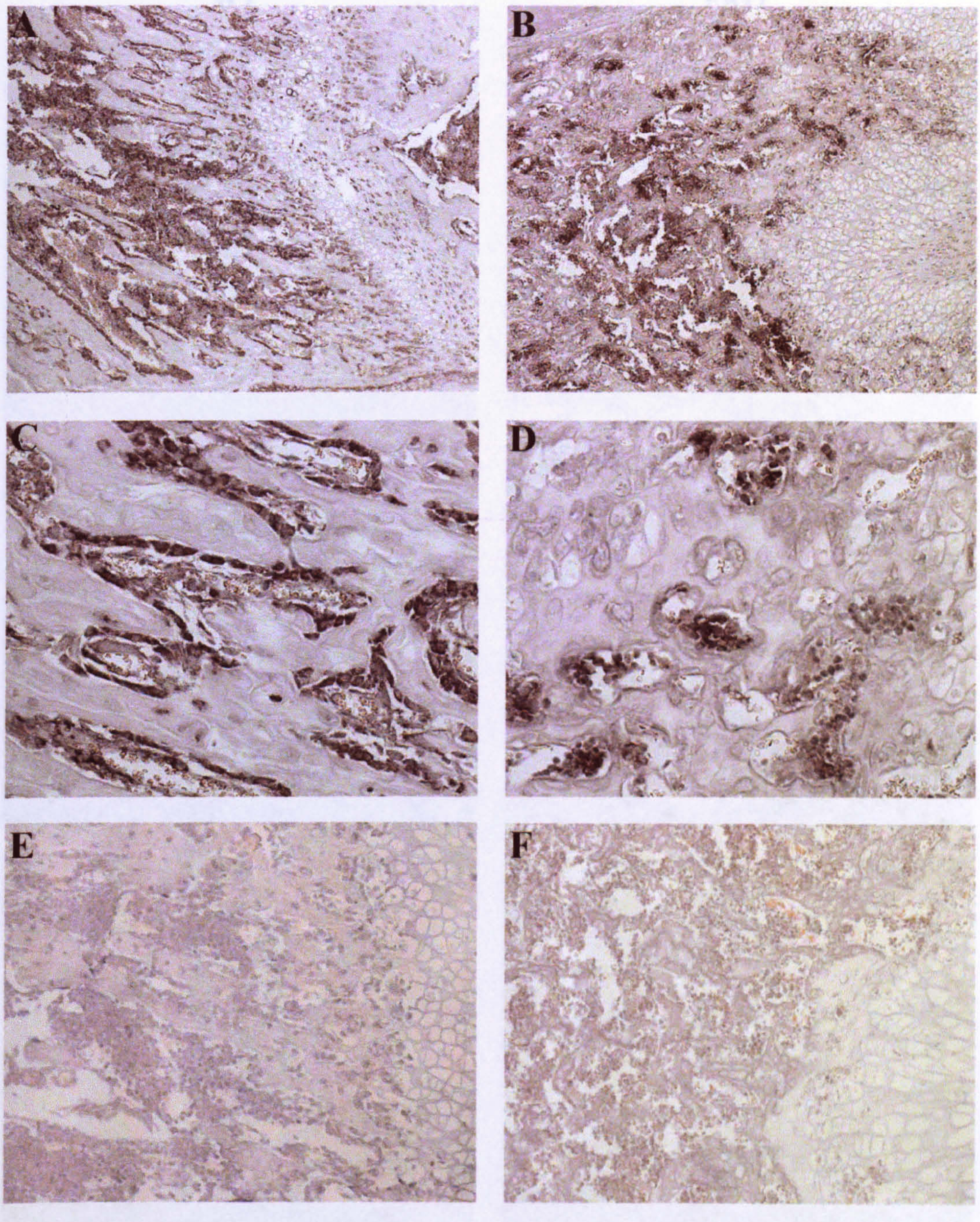



**Fig.3.2.1 Digoxygenin *in situ* hybridisation of *Cbfa-1* expression in long bones of wild-type and *c-fos* knockout mice.** In wild-type long bones, *Cbfa-1* is expressed in osteoblasts and prehypertrophic chondrocytes (A,C). *Cbfa-1* mRNA is also similarly detected in the mutant long bones (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

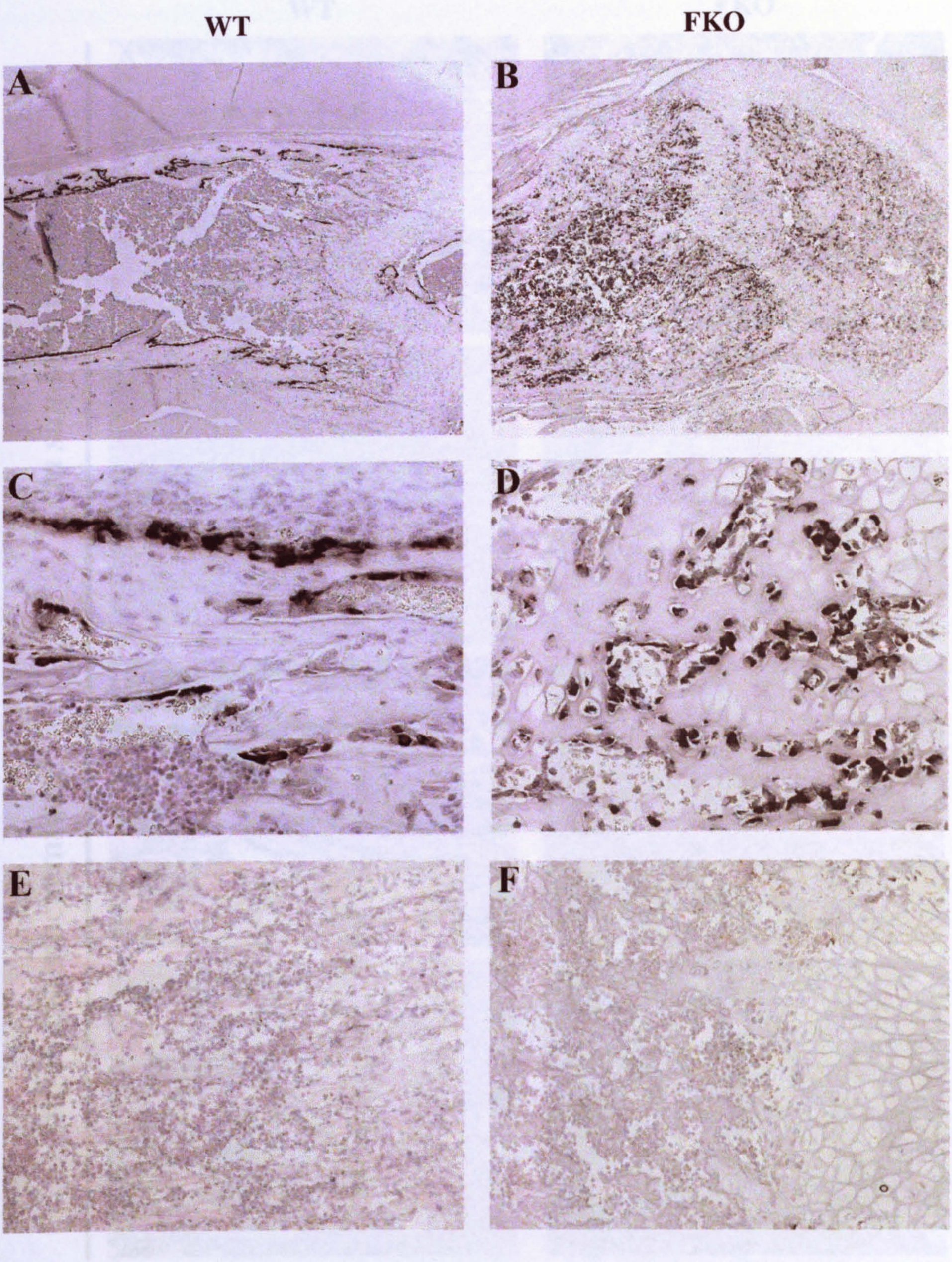



**Fig.3.2.2 Digoxygenin *in situ* hybridisation of *osteocalcin* expression in long bones of wild-type and *c-fos* knockout mice.** In wild-type long bones, strong expression of *osteocalcin* is observed in osteoblasts (A,C). *Osteocalcin* mRNA is also strongly expressed in osteoblasts in mutant long bones (B,D). E and F show sense controls. Original magnification: A, B ×5; C and D ×40; E, F ×20.

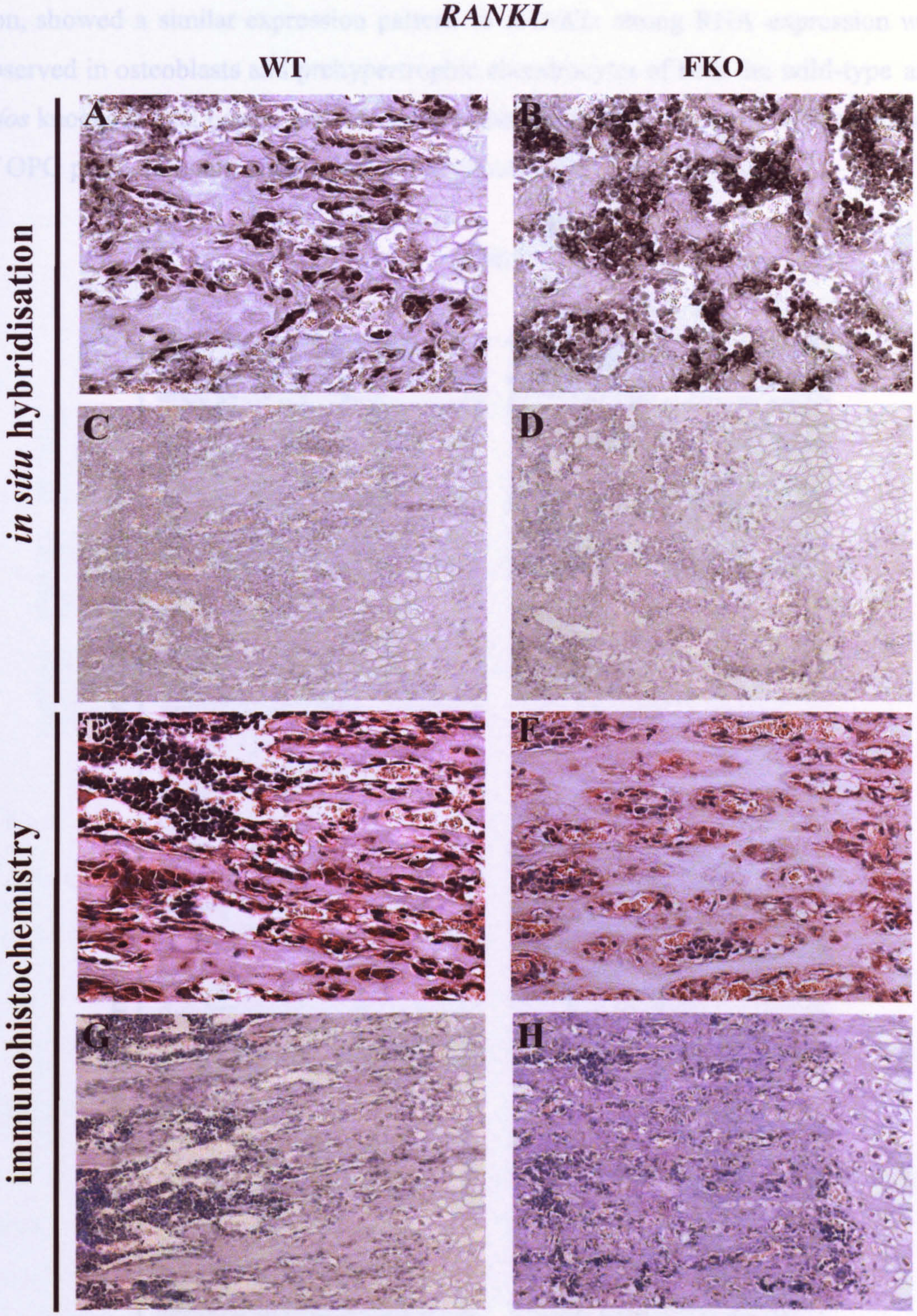



**Fig.3.2.3 Digoxygenin *in situ* hybridisation and immunolocalisation of *RANKL* expression in long bones of wild-type and *c-fos* knockout mice.** Strong expression of *RANKL* mRNA (A) and protein (E) is observed in osteoblasts of wild-type long bones. *RANKL* mRNA (B) and protein (F) are also highly expressed in the mutant osteoblasts. C,D and G,H show sense and negative controls for the *in situ* hybridisation and immunolocalisation studies, respectively. Original magnification: A, B, E, F ×40; C, D, G, H ×40.

*In situ* hybridisation of *OPG*, the potent inhibitor of osteoclast differentiation, showed a similar expression pattern to *RANKL*: strong RNA expression was observed in osteoblasts and prehypertrophic chondrocytes of both the wild-type and *c-fos* knockout long bones, and immunolocalisation studies confirmed the expression of OPG protein in both wild-type and knockout bones (Fig.3.2.4 A-F).

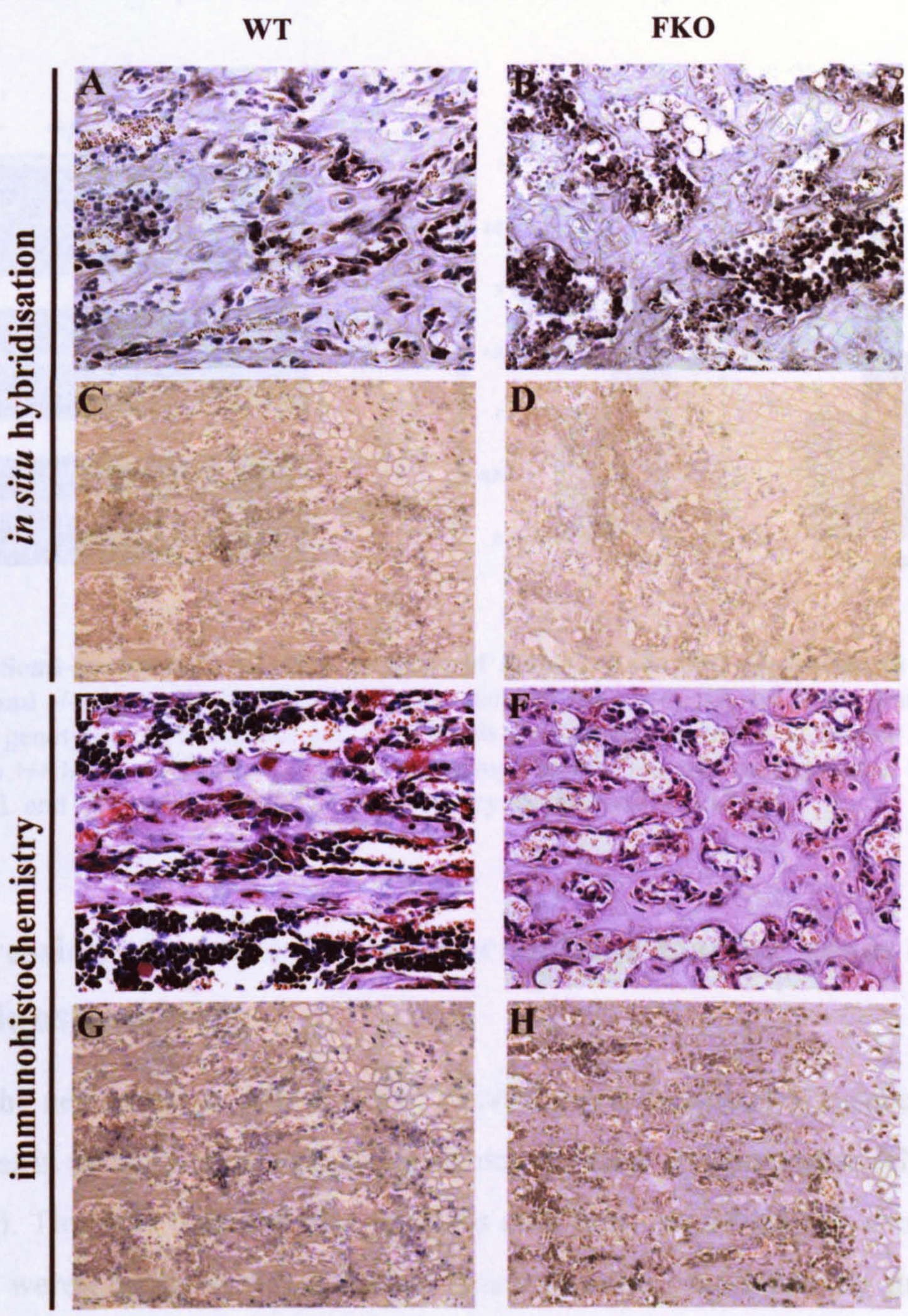



**Fig.3.2.4 *In situ* hybridisation and immunolocalisation of *OPG* expression in long bones of wild-type and *c-fos* knockout mice.** In wild-type long bones, strong expression of *OPG* mRNA (A) and protein (E) is observed in osteoblasts. *OPG* mRNA (B) and protein (F) are also strongly expressed in the mutant osteoblasts. C,D and G,H show sense and negative controls for the *in situ* hybridisation and immunolocalisation studies, respectively. Original magnification: A, B, E, F ×40; C, D, G, H ×20.

As the *in situ* and immunohistochemical analyses are not quantifiable, semi-quantitative RT-PCR of *RANKL* and *OPG* were performed with total RNA isolated from *c-fos* +/+, +/- and -/- limbs. The expression levels of both *RANKL* and *OPG* were upregulated in *c-fos* knockout limbs. Additionally, the expression of *OPG* was upregulated to a lesser extent than that of *RANKL* (Fig.3.2.5). Taken together, it is suggested that the defects in osteoclast differentiation are unlikely to be caused by gross alterations in the production of RANKL and OPG by osteoblasts.

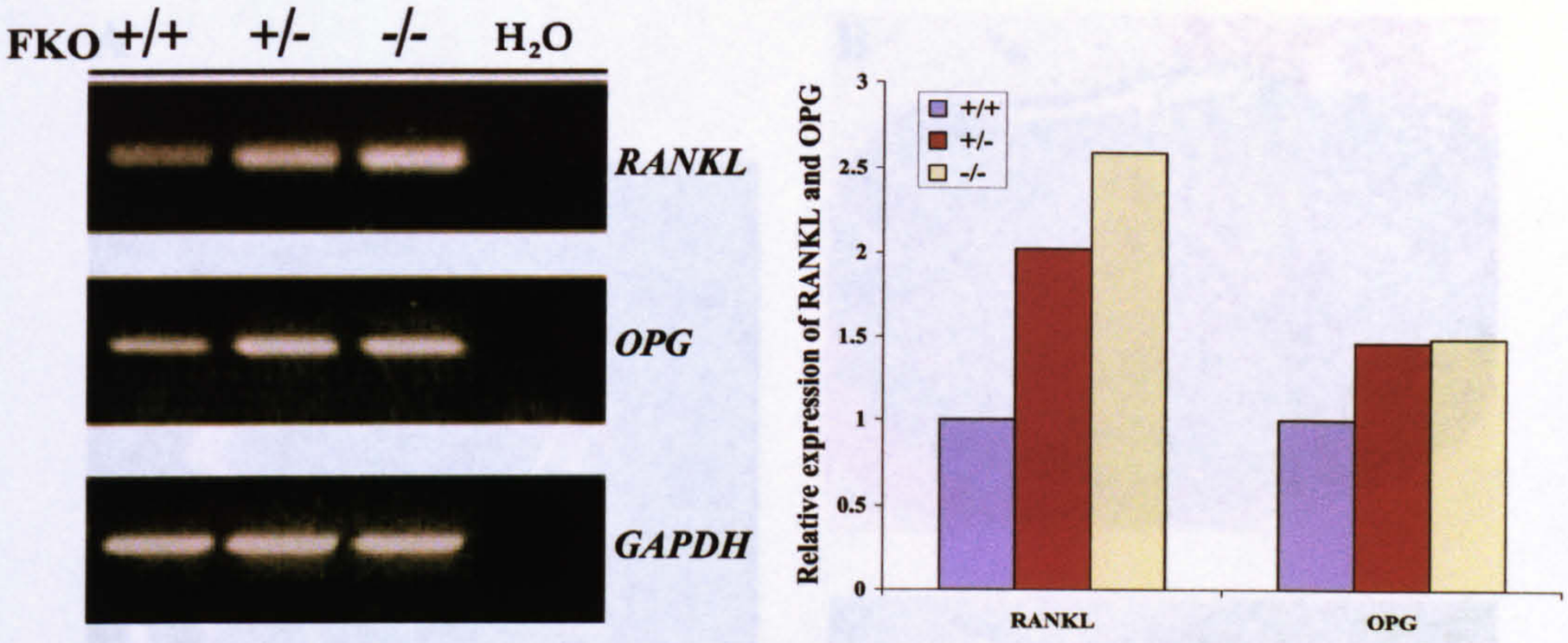



**Fig.3.2.5 Semi-quantitative RT-PCR analysis of *RANKL* and *OPG* expression in *c-fos* +/+, +/- and -/- mouse limbs.** Total RNA was isolated from 5-month old limbs from the indicated genotypes. Both *RANKL* and *OPG* levels are upregulated in the knockout limbs relative to +/+ limbs. *GAPDH* was used as a loading control. The relative expression levels of RANKL and OPG were analysed by densitometry, normalised to GAPDH.

## 3. 4 Expression of osteoclast and macrophage marker genes in *c-fos* transgenic osteosarcomas

In the next series of experiments, I investigated the status of osteoclasts and macrophages in mice overexpressing c-*fos*, which develop osteosarcomas (Grigoriadis et al., 1993). This was done because previous data from our laboratory showed that osteoclasts were present in c-*fos*-induced osteosarcomas predominantly during the phase of rapid tumour growth (El-Emir and Grigoriadis, unpublished). Moreover, osteosarcomas in double c-*fos*/c-*jun* transgenic mice develop faster and have more osteoclasts, while c-*fos* transgenic mice crossed with c-*fos* knockout mice have virtually no tumours (Wang et al., 1995). These results suggest an important role for c-Fos

for not only tumour development, but perhaps also for osteoclast-mediated remodelling within growing osteosarcomas.

Figure 3.3.1 characterises the basic features of osteosarcomas from *c-fos* transgenic mice. These tumours contain large amounts of neoplastic bone which often invades into the bone marrow spaces (Fig.3.3.1 B,C), and which is actively remodelled by numerous cuboidal osteoblasts and multinucleated osteoclasts (Fig.3.3.1 D,E).

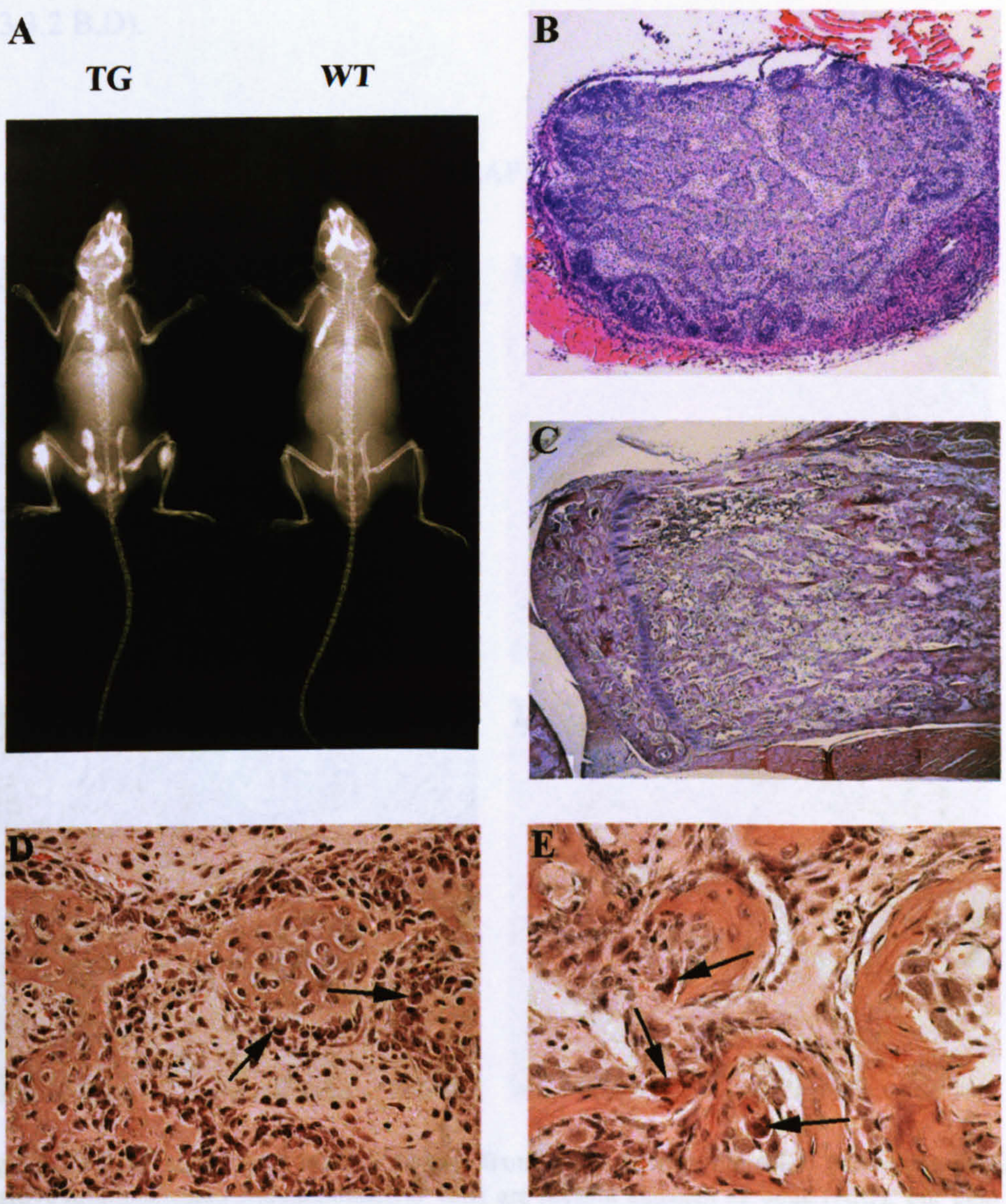



**Fig.3.3.1 Radiographic and histological analysis of osteosarcomas in c-Fos transgenic mice**. X-ray shows calcified tumours in c-Fos transgenic skeleton (A, arrows). Haematoxylin+Eosin staining shows that tumours contain large amounts of bone (B) which often invade into the bone marrow space (C). Detailed histological analysis shows active bone remodelling with numerous cuboidal osteoblasts (D, arrows) and multinucleated osteoclasts (E, arrows). Original magnification: B, C ×5; D, E ×40.

In all the following *in situ* studies, the expression of the relevant marker genes was examined in two basic areas of the tumours: areas containing bone, and areas in the periphery of the tumours which are more fibroblastic in nature, although the exact characteristics of the cell types present in the fibrous regions are not known.

Histochemical staining for TRAP activity showed that many TRAP positive multinucleated and mononuclear cells were present in the tumours, mostly situated in lacunae on bone surfaces (Fig.3.3.2 A,C). In addition, many TRAP-positive mononuclear cells were observed in fibroblastic areas without any evidence of bone present (Fig.3.3.2 B,D).

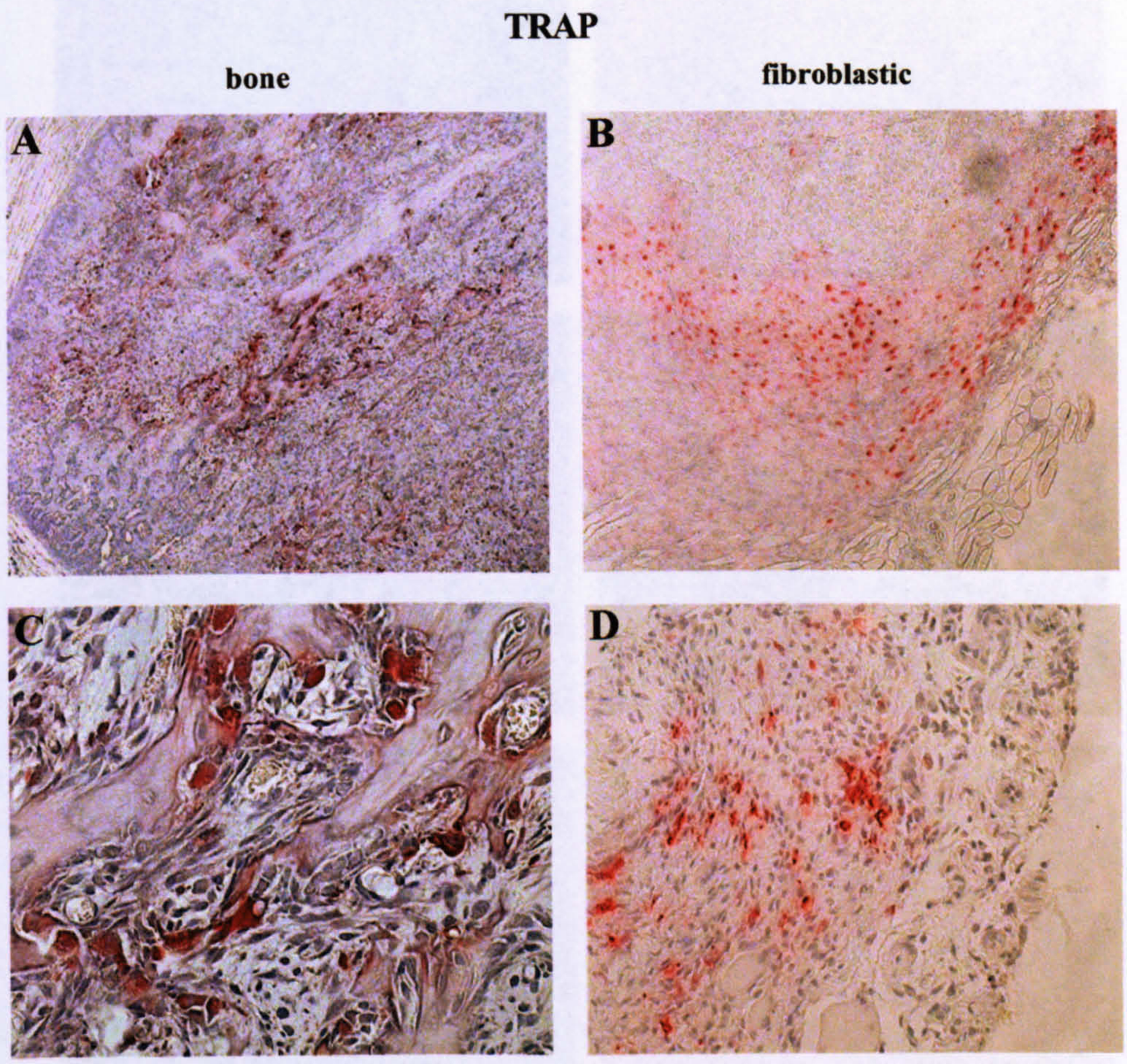



**Fig.3.3.2 TRAP-staining of tumour tissues from c-Fos transgenic mice.** Numerous TRAP-positive multinucleated and mononuclear cells are present adjacent to bone (A,C), but also in the fibroblastic areas of the tumour margin not containing any bone (B,D). Original magnification: A, B ×10; C, D ×40.

This was surprising as osteoclasts normally form in areas adjacent to bone surfaces. In order to further characterise those TRAP-positive cells, the expression of other osteoclast maker genes was investigated. Analysis of *Cathepsin K* and *MMP-9* expression showed very strong expression in all multinucleated osteoclasts and many mononuclear precursors in bone-containing areas within the tumours, and, as observed with TRAP staining, mononuclear cells in fibroblastic areas also expressed these two osteoclast marker genes (Figs.3.3.3 A-D, and 3.3.4 A-D).

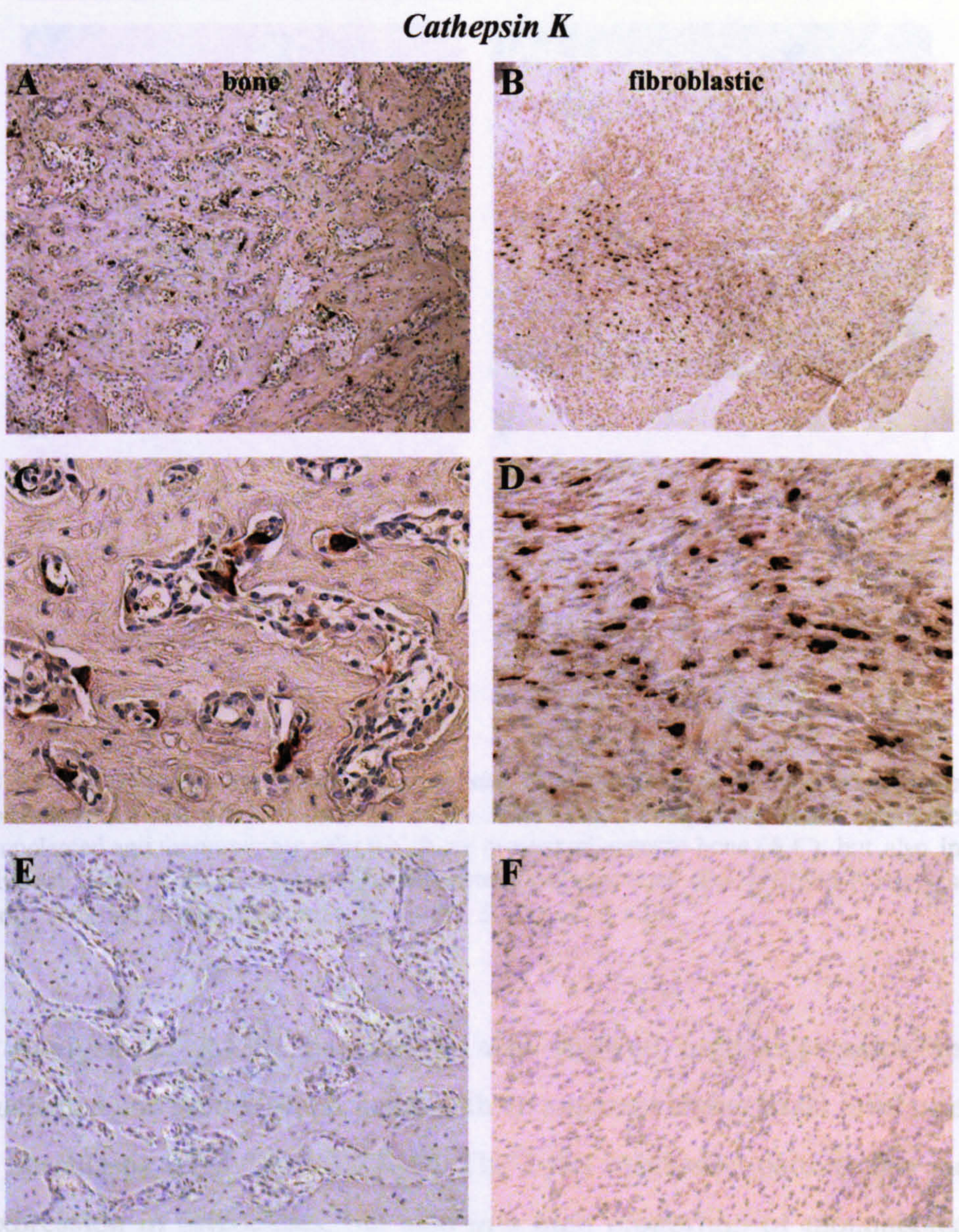



**Fig.3.3.3 Digoxygenin *in situ* hybridisation of *cathepsin K* expression in tumour tissues from c-Fos transgenic mice.** *Cathepsin K* is expressed by numerous multinucleated and mononuclear cells which are present adjacent to bone (A,C), but also in fibroblastic areas of the tumour margin not containing any bone (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

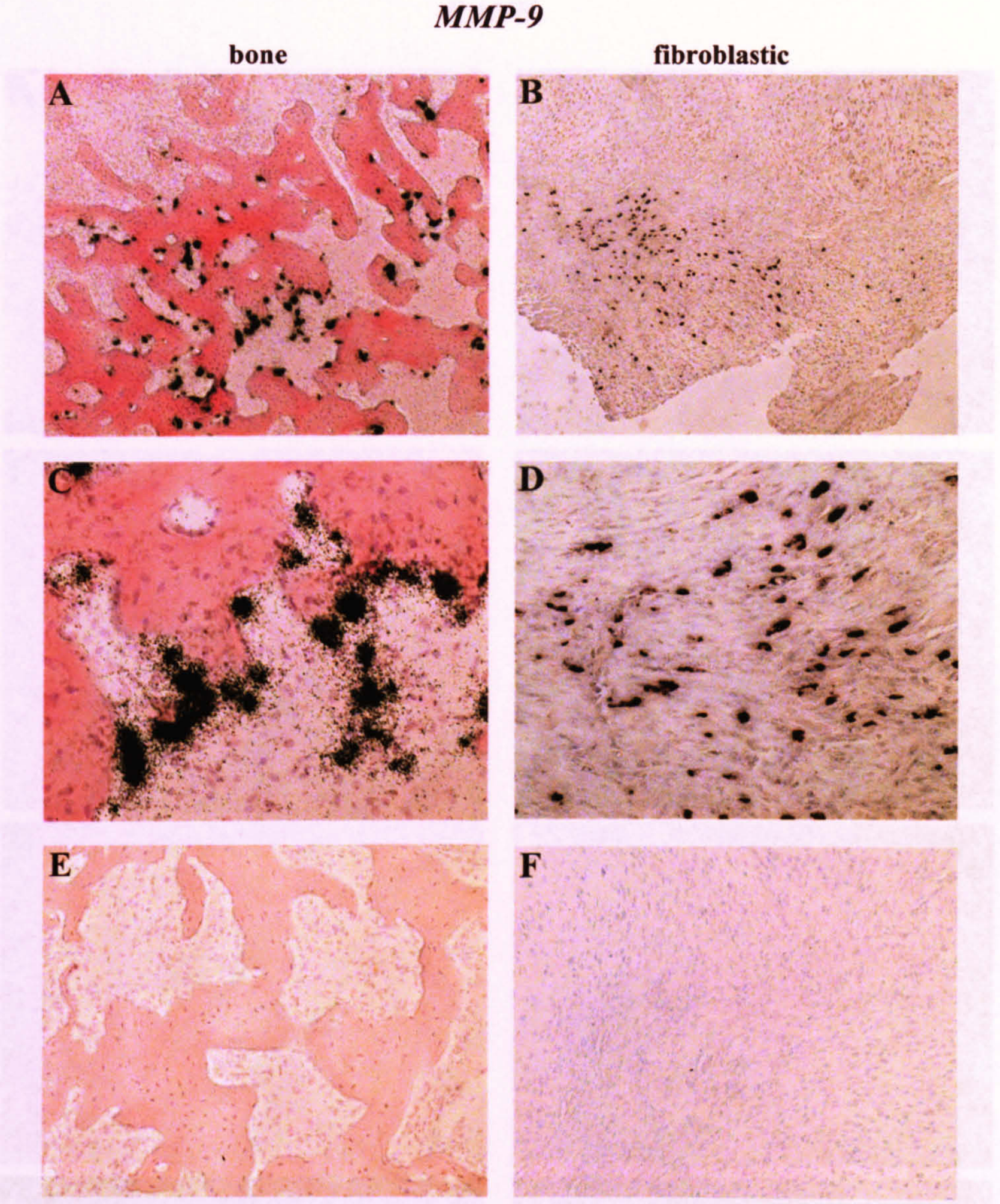



**Fig.3.3.4 Digoxygenin and radioactive *in situ* hybridisation of *MMP-9* expression in tumour tissues from c-Fos transgenic mice.** *MMP-9* is strongly expressed by numerous multinucleated and mononuclear cells which are present adjacent to bone (A,C), but also in the fibroblastic areas of the tumour margin not containing any bone (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

Analysis of *RANK* expression also showed high expression in both multinucleated and mononuclear cells, with an apparent higher basal level of expression in fibroblastic areas (Fig.3.3.5 A-F). The c-*fms* expression pattern also indicated high expression in osteoclasts, but also in a high proportion of mononuclear cells (Fig.3.3.6 A-D). Immunostaining for F4/80 antigen showed large numbers of F4/80-positive macrophages were present in bone tumours, while multinucleated osteoclast were negative (Fig.3.3.7 A,B).

***RANK***

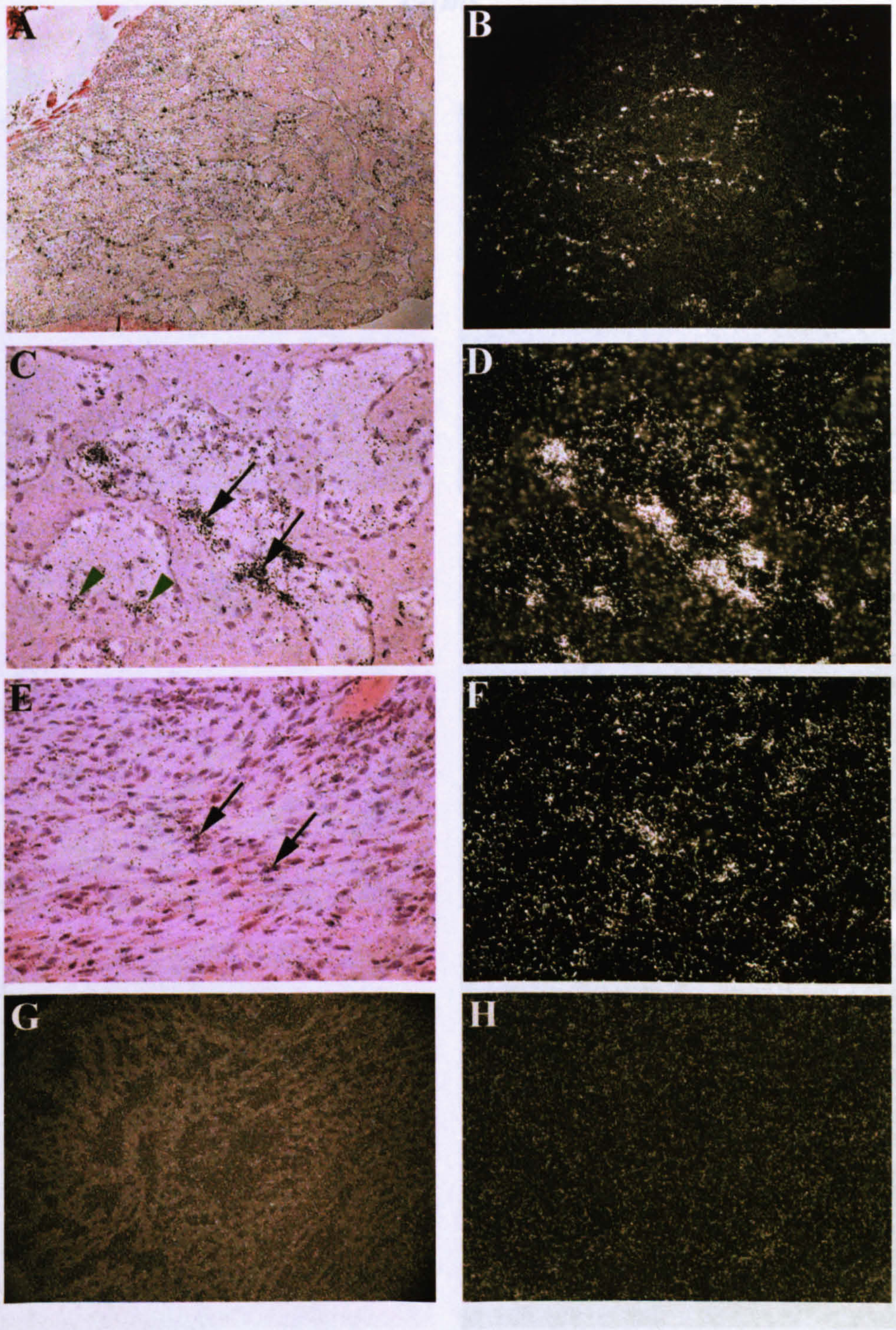



**Fig.3.3.5 Radioactive *in situ* hybridisation of *RANK* expression in tumour tissue from c-Fos transgenic mice.** Strong expression of *RANK* is observed in numerous multinucleated osteoclasts (A,C, arrows) and mononuclear precursors (A,C, arrowheads). *RANK* is also expressed by cells in the fibroblastic areas of the tumour margin not containing any bone (E, arrows). G (bone areas) and H (fibroblastic areas) show sense controls. A, C, E bright field; B, D, F-H dark field. Original magnification: A, B, G ×5; C-F, H ×40.

*c-fms*

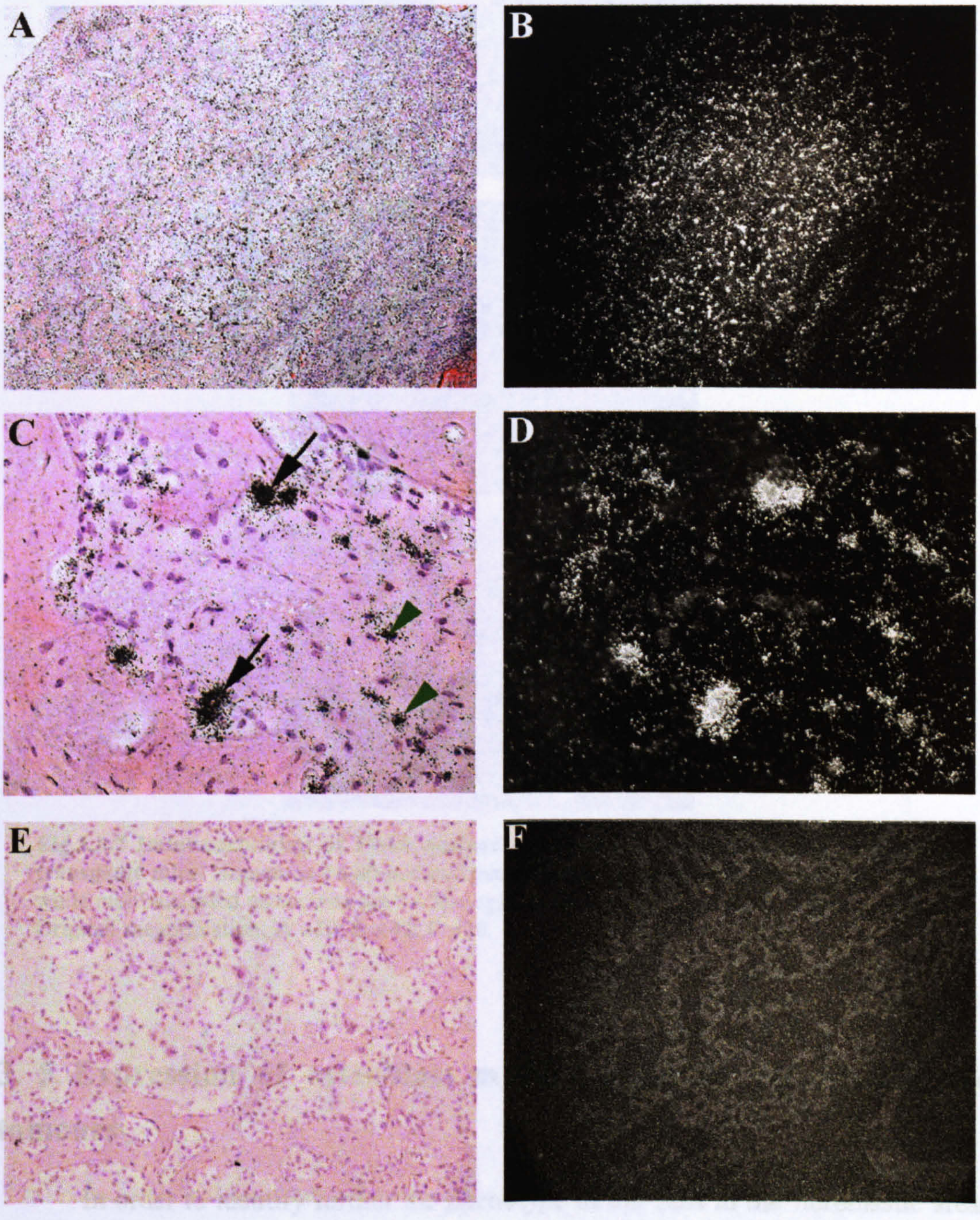



**Fig.3.3.6 Radioactive *in situ* hybridisation of *c-fms* expression in tumour tissues from c-Fos transgenic mice.** c-Fms is expressed by numerous multinucleated cells (A,C, arrows) and mononuclear cells (A,C, arrowheads). E and F show sense controls. A, C, E, bright field; B, D, F dark field. Original magnification: A, B, F ×5; C, D ×40; E ×20.

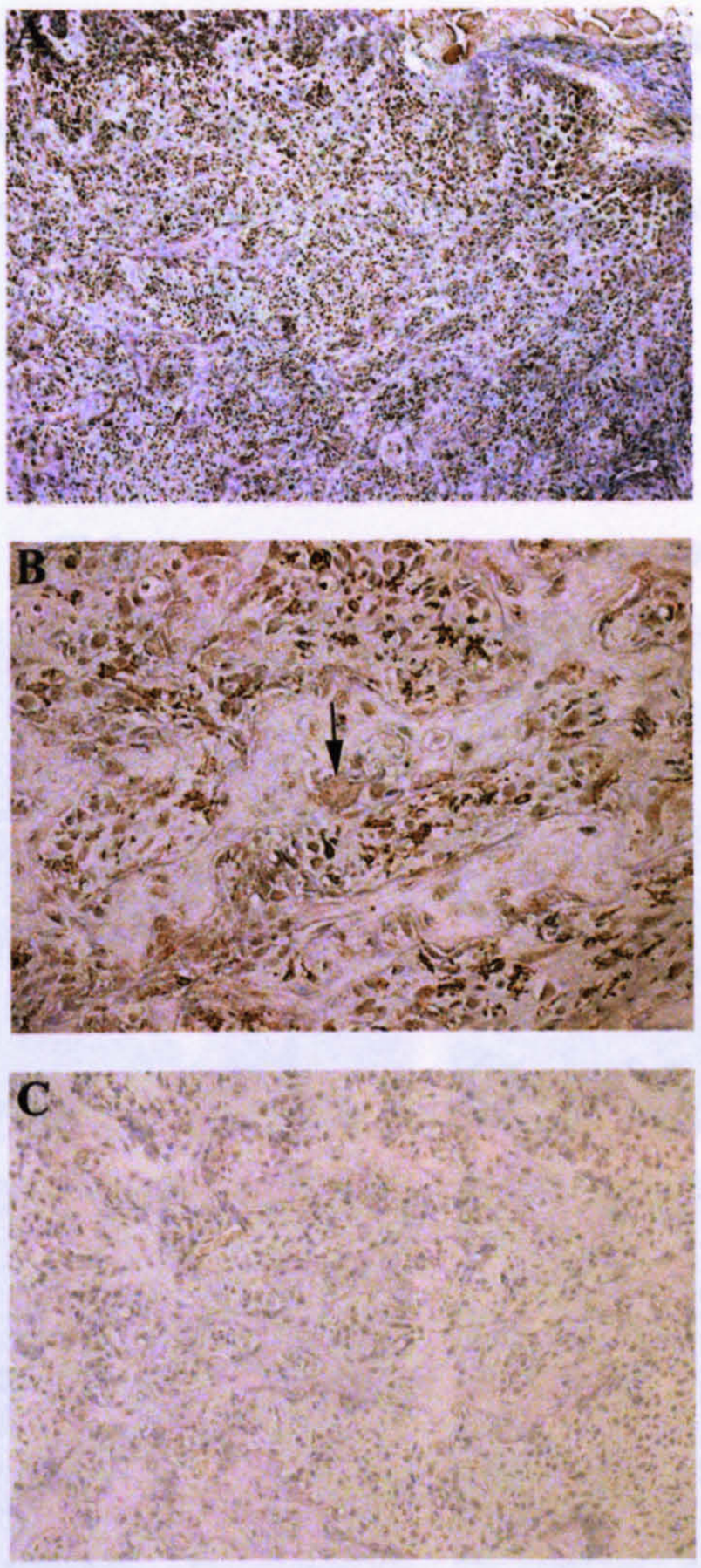



**Fig.3.3.7 Immunostaining of F4/80 antigen expression in tumour tissues from c-Fos transgenic mice.** Numerous F4/80-positive macrophages are present in the tumours (A,B), while multinucleated osteoclasts are F4/80-negative (B, arrow). C shows a negative control. Original magnification: A ×10; B ×40; C ×20.

## 3. 5 Expression of osteoblast marker genes in c-*fos* transgenic tumours

In order to identify further the phenotype of the cells in the fibroblastic areas of the tumours, and to find out the possible reasons for osteoclast formation in these areas, *in situ* hybridisation of osteoblast marker genes and genes that are critical for osteoclast differentiation were performed in tumour tissues.

*In situ* hybridisation of *Cbfa-1* showed strong expression not only in transformed osteoblasts, but also in fibroblastic cells present at the tumour margins

(Fig.3.4.1). Hybridisation of adjacent sections with *osteocalcin* showed that while *osteocalcin* was strongly expressed in definitive cuboidal osteoblast-like cells, it was not expressed in fibroblastic cells (Fig.3.4.1 C,D). These data suggested that the cells in the fibrous regions were potentially pre-osteoblasts, which had not undergone terminal differentiation.

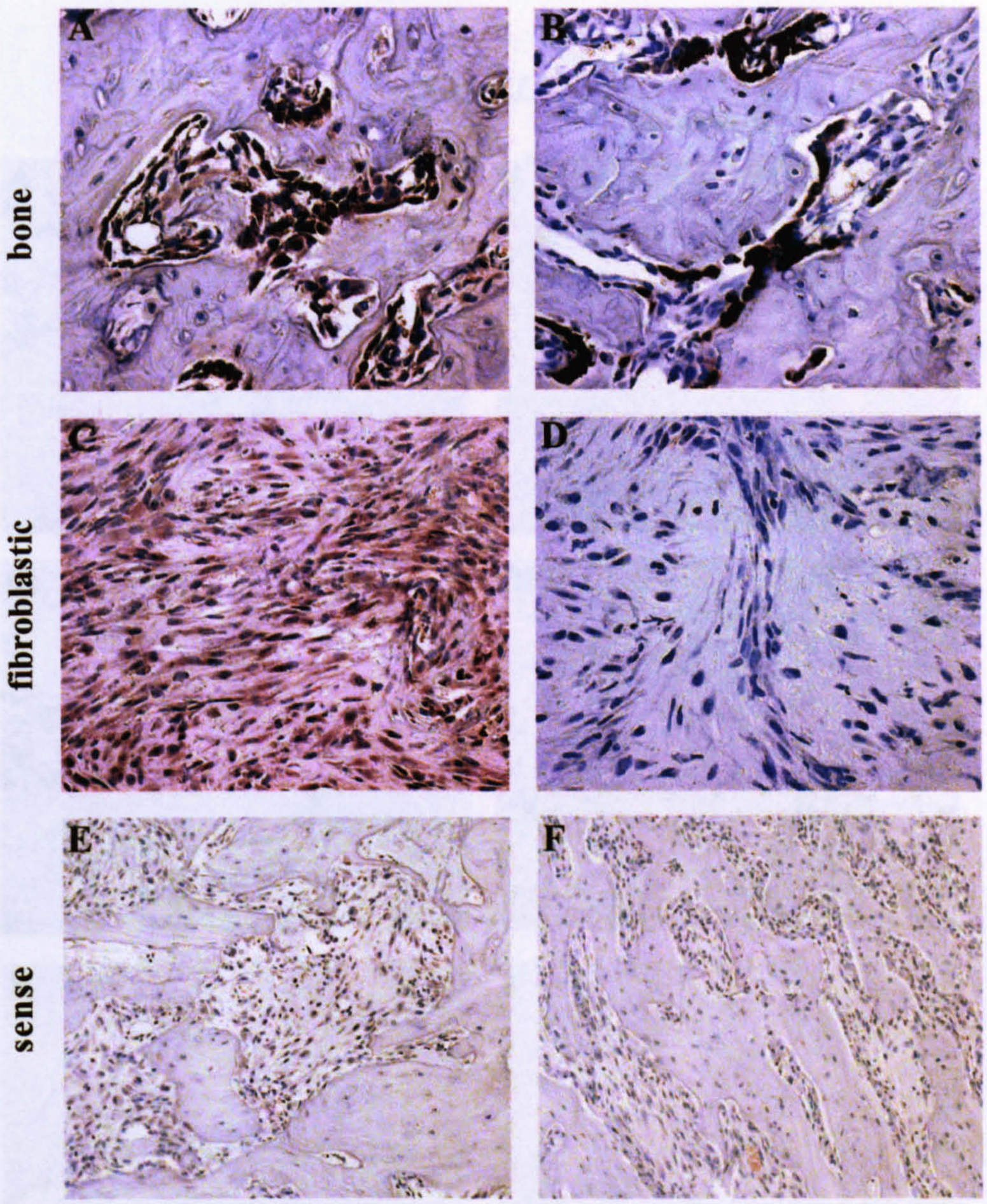



**Fig.3.4.1 Digoxygenin *in situ* hybridisation of *Cbfa-1* and *osteocalcin* expression in tumour tissues from c-Fos transgenic mice.** *Cbfa-1* is expressed in transformed osteoblast-like cells (A) and fibroblastic cells (C), while *osteocalcin* is only expressed in osteoblast-like cells (B) and not in fibroblastic cells (D). E and F show negative controls. Original magnification: A-D ×40; E, F ×20.

To investigate whether these cells were capable of supporting osteoclast differentiation, *RANKL* and *OPG* expression were analysed in adjacent sections. *In situ* hybridisation of *RANKL* showed strong expression not only in transformed osteoblast- like cells but also in fibroblastic cells present within the tumours at sites not in close proximity to bone (Fig.3.4.2 A-D). Similarly, *OPG* was strongly expressed not only in osteoblast- like cells but also in fibroblastic cells present at the tumour margins (Fig.3.4.3 A-D). Thus, these results suggest that the cells within fibroblastic area have the ability to support osteoclast differentiation.

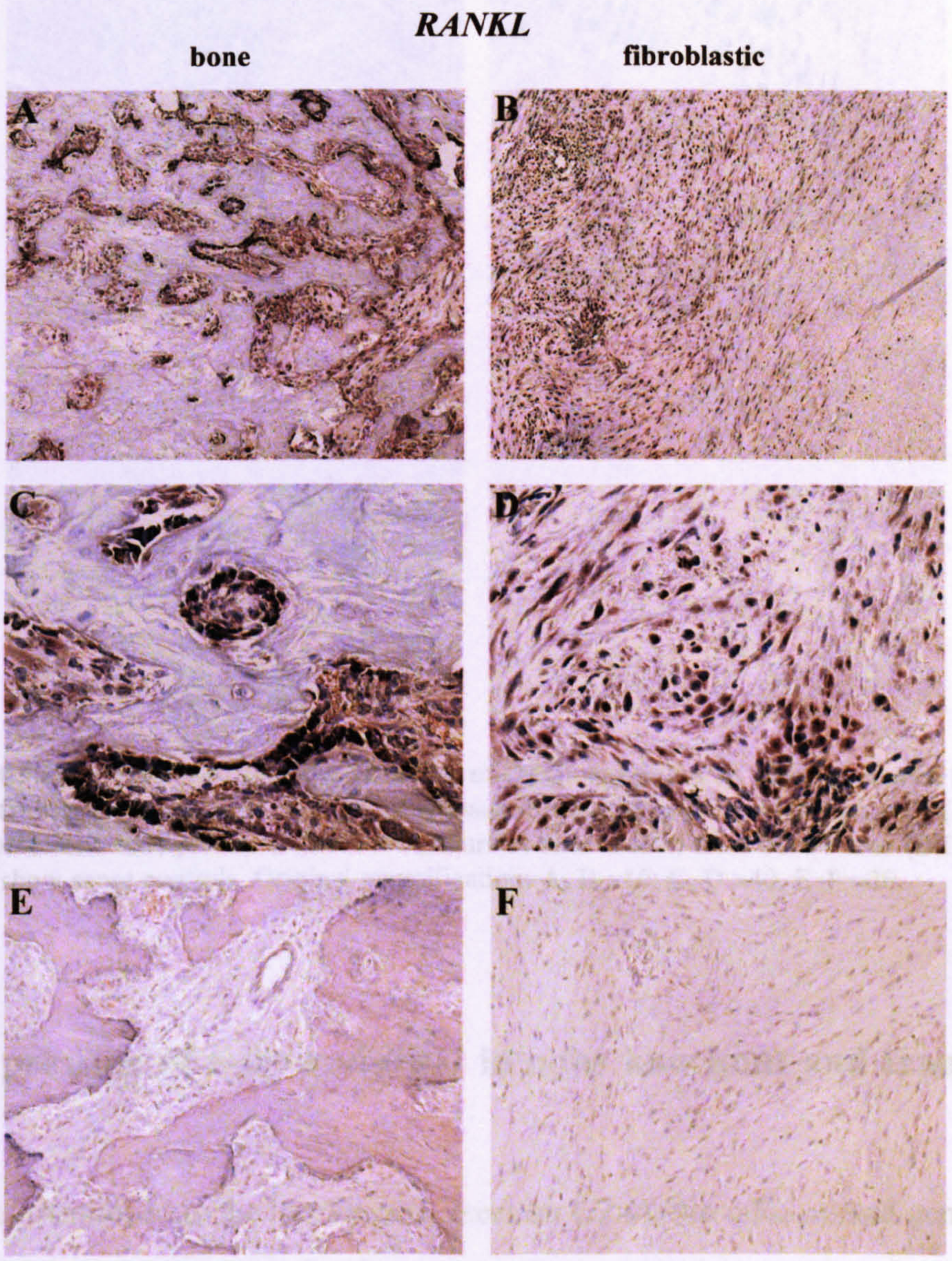



**Fig.3.4.2 Digoxygenin *in situ* hybridisation of *RANKL* expression in tumour tissues from c-Fos transgenic mice.** *RANKL* is highly expressed in transformed osteoblast-like cells (A,C), and also in fibroblastic cells present within tumours at sites distant from bone tissues (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40; E, F ×20.

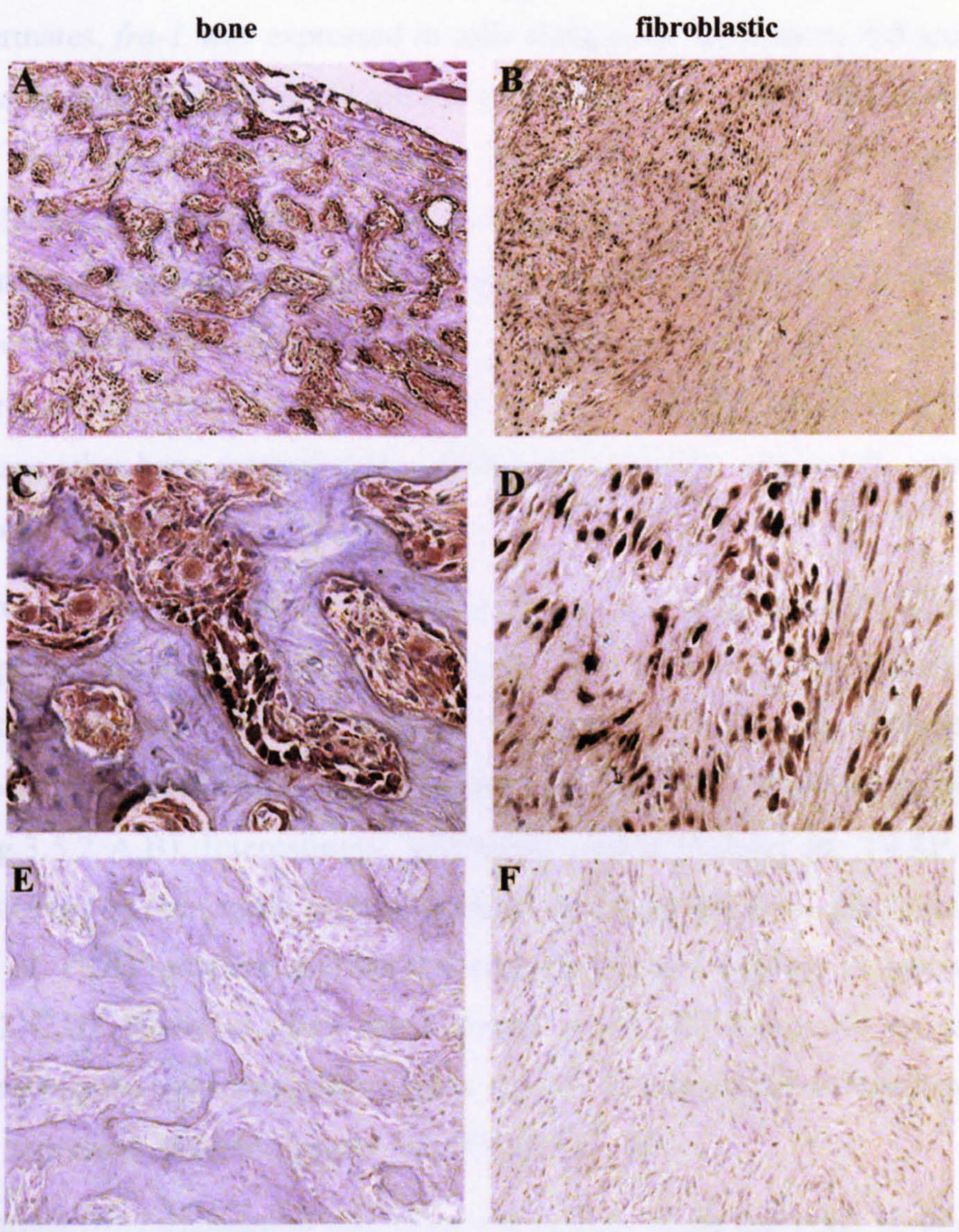



**Fig.3.4.3 Digoxygenin *in situ* hybridisation of *OPG* expression in tumour tissues from c-Fos transgenic mice.** *OPG* is highly expressed in transformed osteoblast-like cells (A,C) and fibroblastic cells present within the tumours at sites distant from bone tissue (B,D). E and F show sense controls. Original magnification: A, B ×10; C, D ×40. E, F ×20.

## 3. 6 Expression of *c-fos* and *fra-1* in c-*fos* knockout and transgenic mice

As mentioned in the Introduction (section 1.7.4), the c-*fos*-related gene, *fra-1*, is a downstream target gene of *c-fos*, and is also extremely important in osteoclast differentiation. Thus, in this section, the expression of *fra-1* and *c-fos*, were investigated *in situ* in the context of osteoclast differentiation.

I first investigated the expression of *fra*-1 in c-*fos* knockout mice. In wild-type littermates, *fra-1* was expressed in cells along some surfaces of trabecular bone as well as in cells along the distal growth plate (Fig. 3.5.1 A,C). Additional double staining for TRAP activity showed that those cells were TRAP-positive multinucleated osteoclasts and mononuclear precursors (Fig.3.5.1 E). Thus, it appears that *fra*-1 was expressed predominantly in osteoclasts. In c-*fos* knockout long bones, there was overall a greater number of cells expressing *fra-1* (Fig.3.5.1 B,D,F). Since there are no osteoclasts in c-*fos* mutants, these cells are most likely osteoblasts and perhaps other bone marrow cells expressing high levels of *fra*-1 to compensate for the lack of c-*fos*.

In c-*fos* transgenic mice, expression of c-*fos* was analysed using a transgene-specific probe (see section 2.3.2.2 in Materials and Methods). Consistent with previous data, exogenous *c-fos* was strongly expressed in many cells, including transformed osteoblastic cells lining the bone surfaces, in addition to fibroblastic tumour cells (Fig.3.5.2 A,B). Interestingly, additional double staining for TRAP activity showed strong *c-fos* transgene expression in some TRAP-positive cells, although the majority of TRAP-positive multinucleated cells did not express exogenous *c-fos* (Fig.3.5.2 C,D). However, there were always many TRAP-positive mononuclear cells expressing the *c-fos* transgene present next to multinucleated osteoclasts, which probably represent osteoclast precursors (Fig.3.5.2 C,D).

Strong *fra-1* expression was observed in cells along the bone surface of tumour tissues, and in contrast to c-*fos* expression (Fig.3.5.3 A,B), additional double staining for TRAP activity showed that *fra-1* was strongly expressed by TRAP-positive multinucleated osteoclasts and mononuclear precursors (Fig.3.5.3 C,D). This suggests that exogenous *c-fos* affects osteoclast formation by upregulating *fra-1* expression.

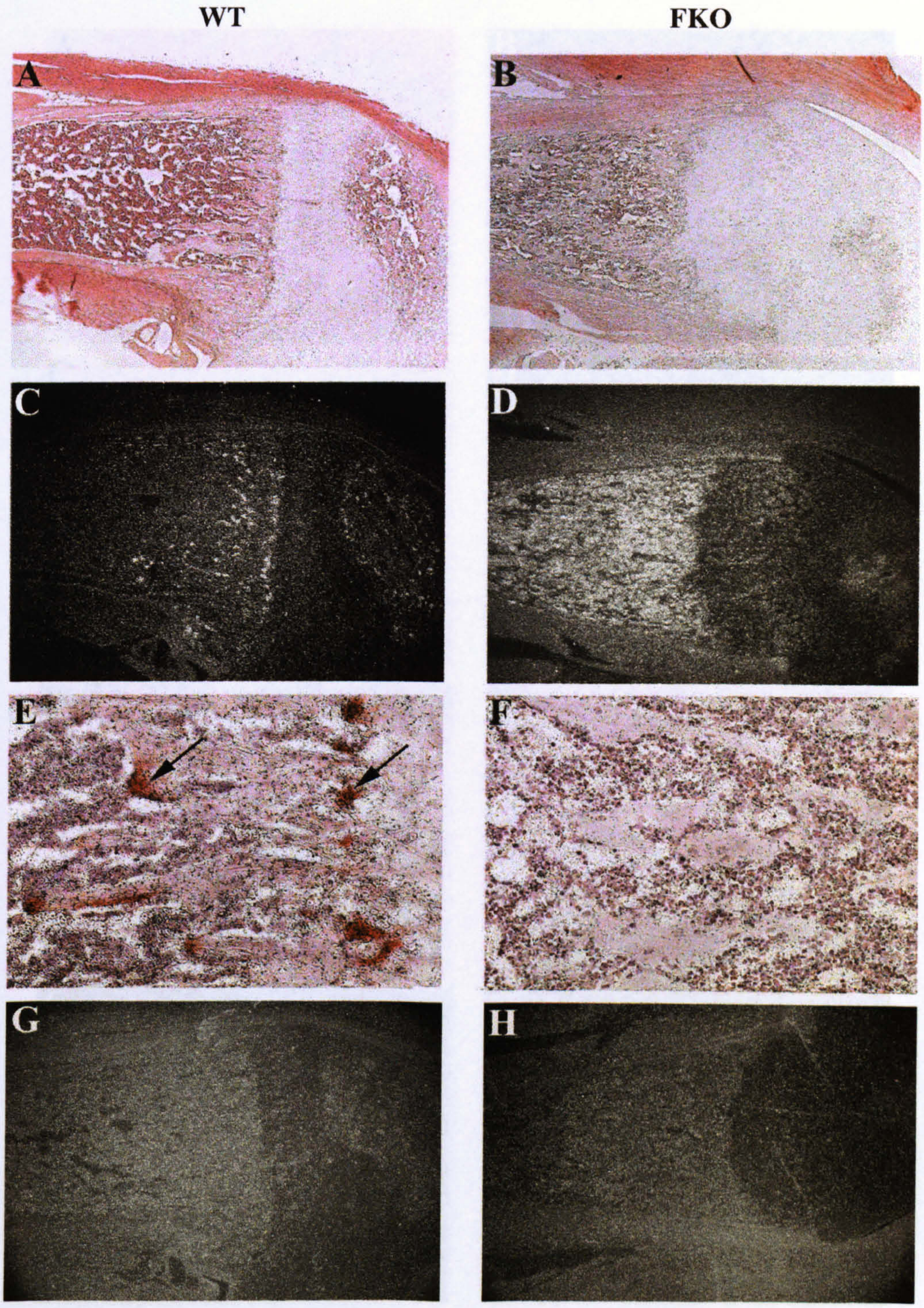



**Fig.3.5.1 Radioactive *in situ* hybridisation of *fra-1* expression in long bones of wild-type and *c-fos* knockout mice.** *Fra-1* is strongly expressed in cells along the bone surfaces in wild-type bones (A,C). More cells express *fra-1* in mutant bones (B,D). Additional double staining for TRAP activity shows cells expressing *fra-1* in wild-type bones are TRAP-positive multinucleated and mononuclear cells (E, arrow), while in mutant bones the *fra-1* positive cells are TRAP-negative mononuclear cells (F). G and H show sense controls. A, B, E, F bright field; C, D, G, H dark field. Original magnification: A-D ×5; E, F ×40; G, H ×20.

## *c-fos* transgene

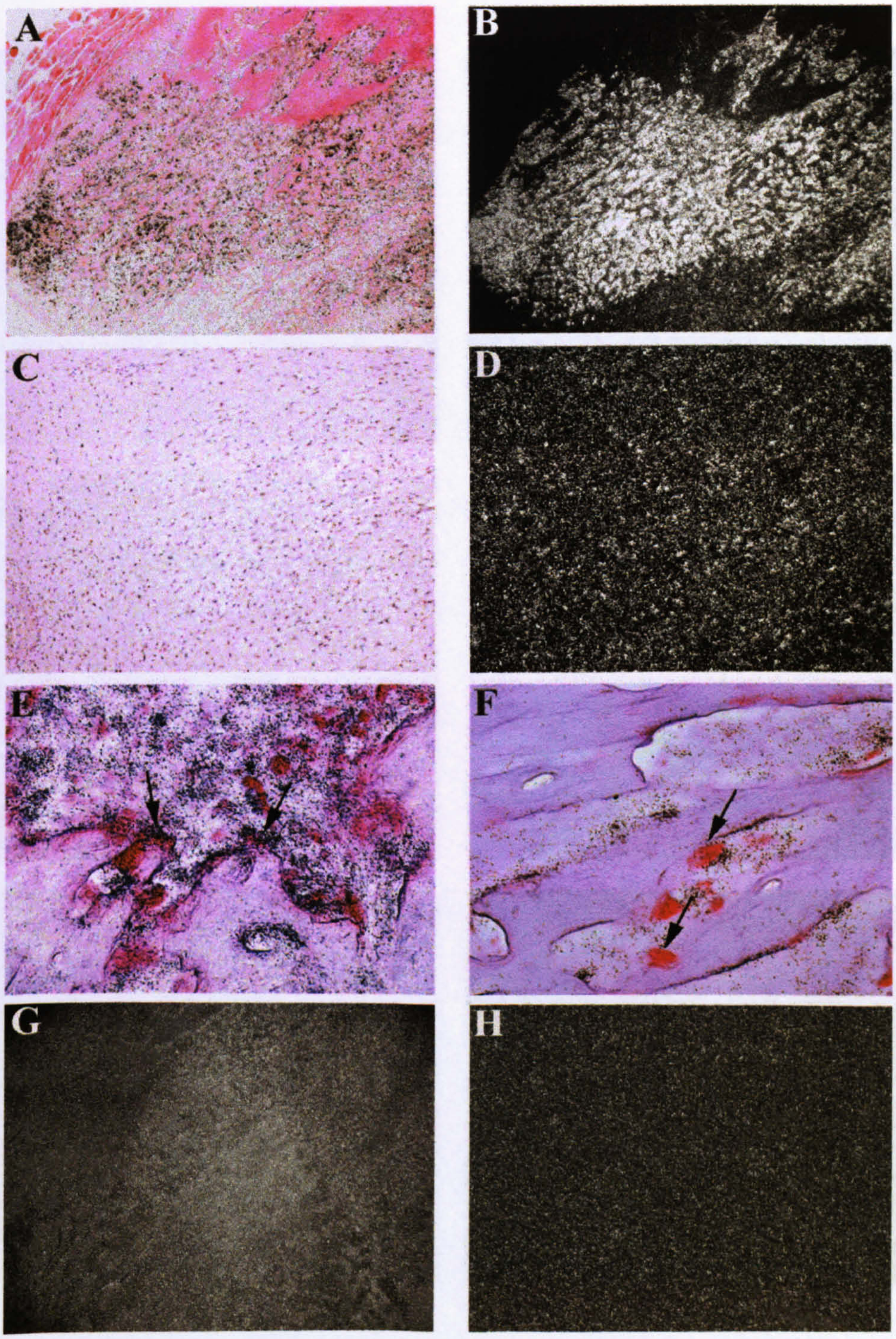



**Fig.3.5.2 Radioactive *in situ* hybridisation of *c-fos* transgene expression in tumour tissues from c-Fos transgenic mice.** Osteoblasts (A,B) and transformed fibroblastic cells (C,D) express high levels of the *c-fos* transgene. Additional double staining for TRAP activity shows strong *c-fos* transgene expression in some TRAP (+) cells (E, arrows), although the majority of TRAP (+) multinucleated cells are negative (F, arrows). G (bone areas) and H (fibroblastic areas) show sense controls. A, C, E, F, bright field; B, D, G, H, dark field. Original magnification: A, B, D, G, H ×5; C, E, F ×40.

***Fra-1***

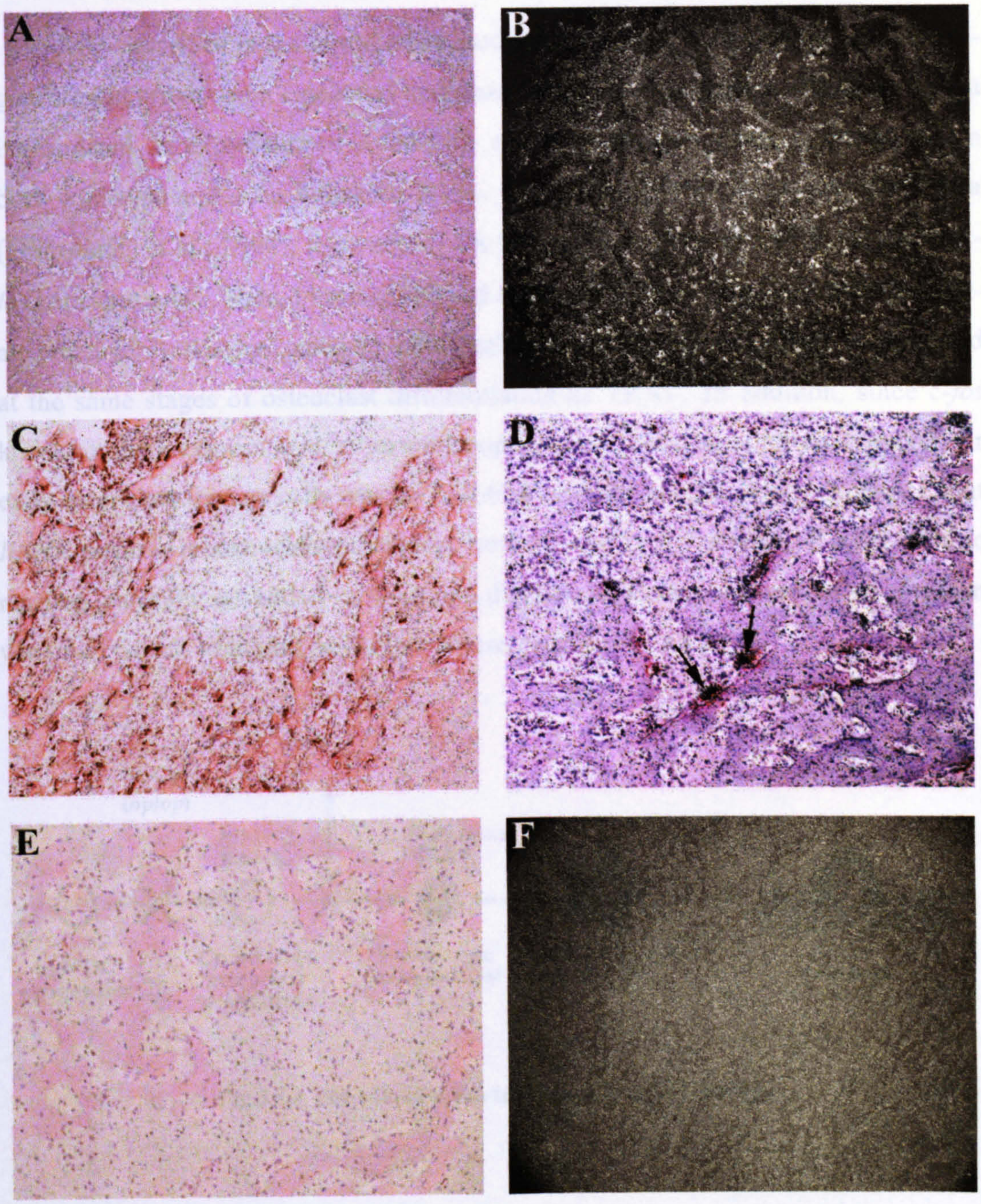



**Fig.3.5.3 Radioactive *in situ* hybridisation of *fra-1* expression in tumour tissues from c-Fos transgenic mice.** High *fra-1* expression is observed in cells adjacent to bone (A,B). Additional double staining for TRAP activity shows *fra-1* is strongly expressed by TRAP-positive mononuclear and multinucleated cells (C,D, arrows). E and F show sense controls. A, C, D, E, bright field; B, F dark field. Original magnification: A, B, F ×5; C ×10; D ×40; E ×20.

## 3. 7 Discussion

### 3.7.1 Inactivation of c-Fos affects osteoclast but not osteoblast gene expression

As described in the Introduction (section 1.7), c-Fos is crucial for osteoclastogenesis and affects both osteoclast and macrophage differentiation. Therefore I first investigated the expression of osteoclast- and macrophage-specific genes *in situ*, in both wild-type and *c-fos* knockout bones. Consistent with previous published data (Grigoriadis et al., 1994), there are no TRAP positive osteoclasts cells present in c-*fos* knockout mice. Here, I have confirmed that mature osteoclasts and their immediate precursors are absent using an additional marker, *cathepsin K*, which is expressed at the same stages of osteoclast differentiation as TRAP. In addition, since c-*fos* knockout mice express *MMP-9* in a subpopulation of cells, an additional marker for early osteoclast lineage cells was used, *RANK*, and the results clearly indicated that *RANK*-positive mononuclear precursors were present in the absence of c-*fos*. These markers can now be added to a lineage diagram indicating the proposed position where c-Fos acts to inhibit osteoclast differentiation (Fig.3.6.1)

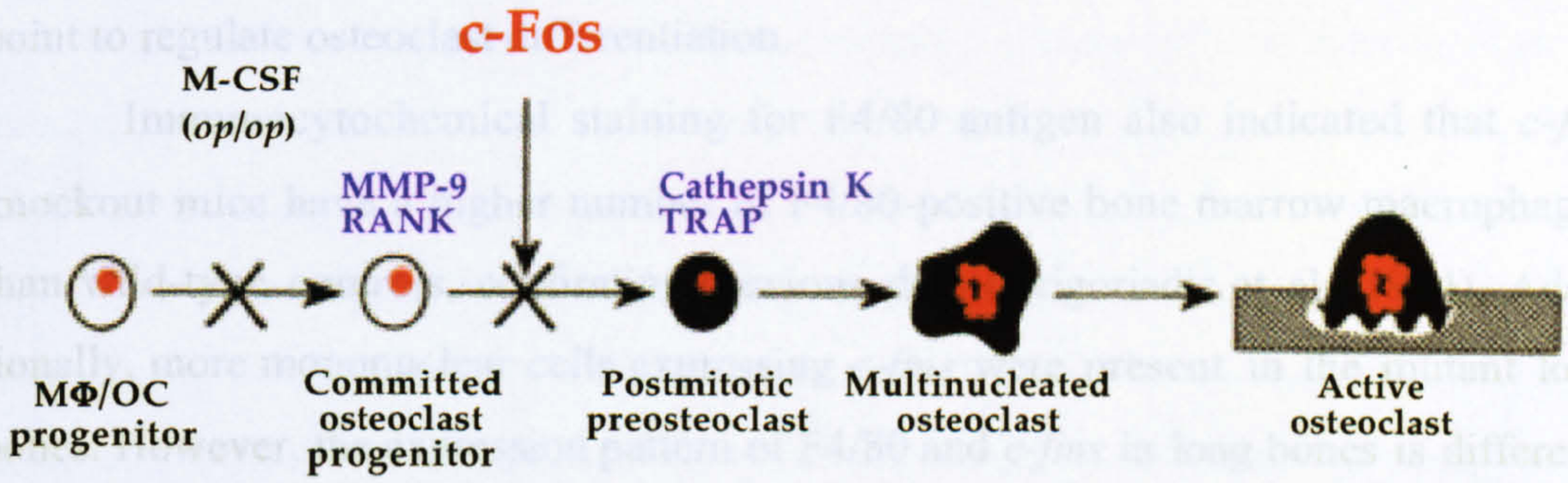



**Fig.3.6.1 The role of c-Fos in osteoclast differentiation**

Among the osteoclastic genes, MMP-9 is known as the earliest marker for the osteoclast lineage (Roodman, 1999). During endochondral bone development, MMP-9 is indispensable for the migration of preosteoclasts to the future bone marrow cavity (Engsig et al., 2000; Reponen et al., 1994). MMP-9 is first expressed by cells outside perichondrium at E15, earlier than TRAP and *c-fms* expression at the same site (Jemtland et al., 1998). At later developmental stages, MMP-9 expression was

most intense at the chondro-osseous margin, while TRAP and *c-fms* expression were more uniform throughout the bone. The distinct expression pattern may represent different osteoclast subpopulations or stages of osteoclastogenesis. MMP-9 positive mononuclear cells are present at the c-Fos mutant growth plate, suggesting that these are osteoclast precursors, and that in the absence of c-Fos, they were unable to further differentiate into TRAP/cathepsin K positive multinucleated osteoclasts.

Similar expression of RANK at the growth plate further confirmed that these cells are osteoclast precursors. RANK, the receptor for RANKL, is widely expressed in the body, but is highly expressed by osteoclasts and their precursors in bone (Hsu et al., 1999). During osteoclastogenesis, RANKL binds RANK on osteoclast precursors, and leads to the activation of NFκB, JNK and c-Fos/AP-1 (Darnay et al., 1998; Kim et al., 1999; Matsuo et al., 2000), which are important signalling molecules and transcription factors for osteoclast differentiation. However, in the absence of c-Fos these other signalling pathways downstream of RANKL/RANK could not rescue osteoclast differentiation, confirming the importance of c-Fos. Nevertheless, the presence of RANK expression in the absence of c-Fos strongly suggests that RANKL-responsive osteoclasts are present, and that c-Fos acts downstream of this point to regulate osteoclast differentiation.

Immunocytochemical staining for F4/80 antigen also indicated that *c-fos* knockout mice have a higher number of F4/80-positive bone marrow macrophages than wild-type controls, confirming previous data (Grigoriadis et al., 1994). Additionally, more mononuclear cells expressing *c-fms* were present in the mutant long bones. However, the expression pattern of F4/80 and *c-fms* in long bones is different: In wild-type mice, F4/80, a mature macrophage marker, was expressed mainly in the bone marrow space, while *c-fms*, the receptor of M-CSF was expressed predominantly in metaphyseal trabecular bone which is similar to osteoclast markers (Hofstetter et al., 1995). In mutant mice, there was an increase in the number of F4/80 positive cells in the marrow space, but this time, c-*fms* expression was also expressed in the bone marrow space, rather than in the metaphysis, suggest that these cells were of the macrophage lineage. Although the number of mature macrophages in c-Fos knockout long bones was increased, there was no difference in the number of ER-MP58-expressing cells, which represent M-CSF-responsive macrophage progenitors (Grigoriadis et al., 1994). Therefore, the different expression pattern of c-*fms* and

F4/80 may suggest that in c-Fos mutant long bones, it is possible that more M-CSF-responsive osteoclast precursors are generated to compensate for the deficiency of osteoclasts, however, in the absence of c-Fos, these cells cannot differentiate further along the osteoclast lineage and may divert to macrophage phenotype instead.

With the exception of c-Fos, other transcription factors, like PU.1, NF-κB and Mitf as well as the production of RANKL and M-CSF by stromal cells, are required for osteoclast/macrophage lineage development. As described in the introduction, PU.1 knockout mice have cell-autonomous effects in the myeloid /monocyte lineage; they do not form osteoclasts or macrophages and develop severe osteopetrosis (Tondravi et al., 1997). The majority of PU.1-/- monocytic precursors detected were at the pre-late marker stage of development, expressing only MOMA-2, ER-MP12, ER-MP58 and ER-MP20 (Henkel et al., 1999). Thus, PU.1 is recognized as the earliest gene controlling osteoclast differentiation.

Regarding NFκB, mice deficient in both the p50 and p52 subunits developed severe osteopetrosis (Franzoso et al., 1997; Iotsova et al., 1997) as they fail to generate mature osteoclasts and B cells. Occasionally, one or two TRAP positive cells were seen in the osteopetrotic limbs, but the intensity of the TRAP staining was very weak. On the other hand, the number of macrophages in bone marrow was increased, which is similar to the c-Fos mutants. In addition, during osteoclastogenesis, both *NFκB* and *c-fos* are activated by M-CSF and RANKL stimulation (Darnay et al., 1999; Matsuo et al., 2000), suggesting that these two transcription factors may share downstream signalling pathways and act similarly in osteoclast lineage, although they are mutually exclusive since one transcription factor cannot compensate for the absence of the other. Furthermore, it has been reported *c-fms* and RANK expressing osteoclast progenitors were present in *NFκB* double knockout spleens (Xing et al., 2002), which I have now shown is similar to the *in situ* results in c-Fos knockout long bones.

Mice deficient in the *mi* (*microphthalmia*) gene, another transcription factor, developed osteopetrosis with dysfunctional immature mononuclear osteoclasts present. By RT-PCR, Roundy et al. showed that *RANK* expression was dramatically enhanced in *mi* mice (Roundy et al., 2003), which suggests that *mi* acts downstream of *c-fos* and NFκB (Luchin et al., 2000).

Mice lacking functional M-CSF, named *op/op* mice, have severe deficiency of osteoclasts, monocytes, and mature macrophages in various tissues (Naito et al., 1991; Wiktor-Jedrzejczak et al., 1992). Mice with a disruption of RANKL gene developed severe osteopetrosis due to complete absence of TRAP-positive mature multinucleated osteoclasts and immature preosteoclasts (Kong et al., 1999). RANK knockout mice developed a similar phenotype as the RANKL knockouts, where the formation and function of dendritic cells and macrophages were unaffected. The distribution of macrophages in mutant spleen was also normal (Dougall et al., 1999).

Taken together, in the cascade of osteoclast differentiation *c-fos* is downstream of *PU.1*, *c-fms* and *RANK*, upstream of *mi*, and similar as *NFκB*, acts at the branch point where osteoclast and macrophage differentiation diverge (Fig.3.6.2)(Karsenty and Wagner, 2002).

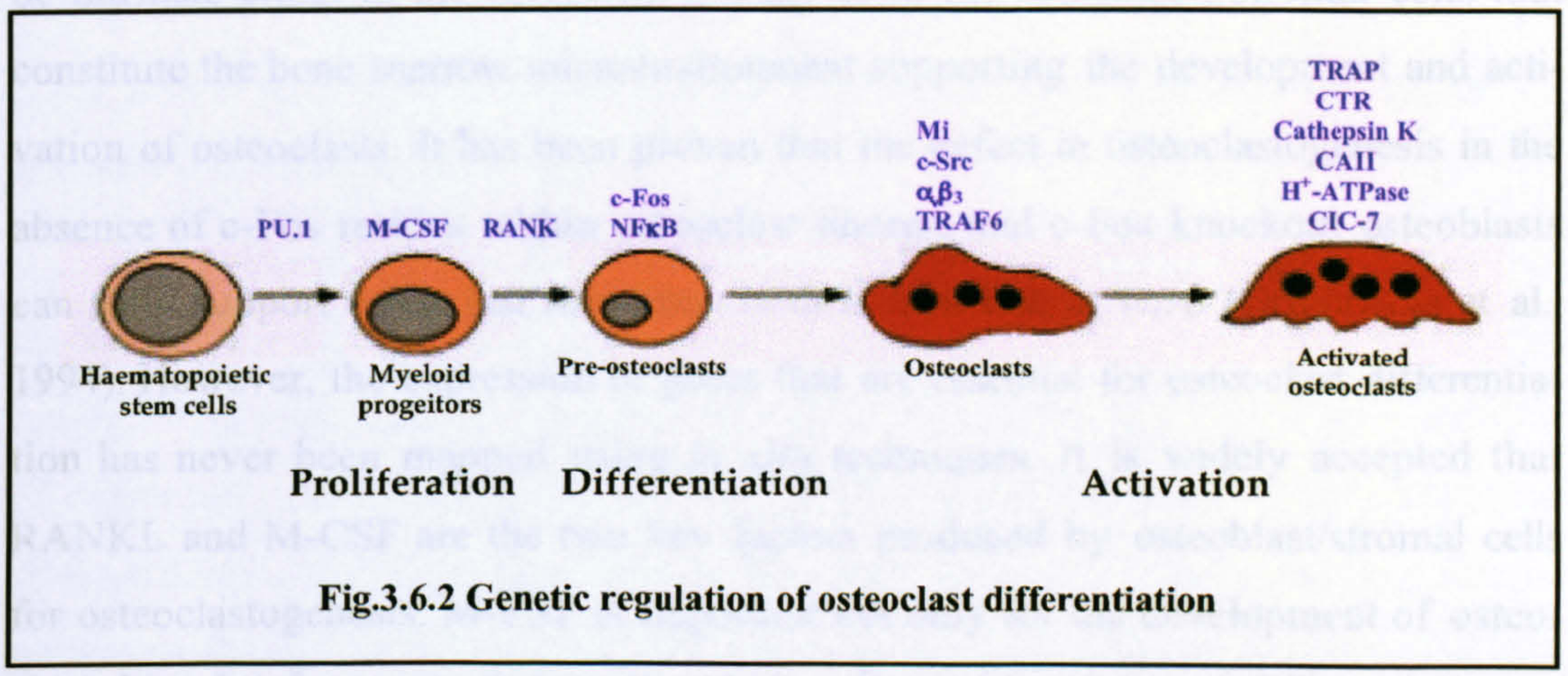



**Fig.3.6.2 Genetic regulation of osteoclast differentiation**

Analysis of the expression of osteoblast marker genes and genes that are critical for osteoclast differentiation was performed in order to investigate the consequences of lacking c-Fos on osteoblasts *in situ*. This was important as previous experiments only examined osteoblast-mediated osteoclastogenesis *in vitro* (see below). Osteoblasts are derived from mesenchymal stem cells, to date, only three osteoblast-specific transcripts have been identified: *Cbfa-1*, its downstream gene *Osx* and *osteocalcin* (Karsenty, 1999; Nakashima et al., 2002). *In situ* hybridisation showed that both genes were strongly expressed by osteoblasts in *c-fos* knockout long bones. *Cbfa-1* is the transcription factor controlling osteoblast development and regulating the expression of bone matrix proteins. Mice lacking *Cbfa-1* developed cartilaginous skeletons due to complete absence of osteoblast differentiation (Komori et al., 1997).

The similar expression of *Cbfa-1* in *c-fos* mutant and wild type long bones suggests that early osteoblast differentiation and function are not affected in the absence of *c-fos*. *Osteocalcin* is a gene expressed only in terminally differentiated osteoblasts, and the secreted molecule it encodes inhibits osteoblast function (Ducy et al., 1996). Previous studies showed that in *op/op*, *tl/tl* osteopetrotic rat mutations, *osteocalcin* mRNA levels were significantly decreased (Shalhoub et al., 1991). In *c-fos* knockouts, the level of *osteocalcin* expression was only slightly lower than that of wild type. Since bone formation is coupled with osteoclastic bone resorption, active osteoblast differentiation and function are likely to be retarded in the absence of osteoclasts. Hence, the decreased osteocalcin expression may be secondary to the defect of bone remodelling in the absence of c-Fos.

Previous studies have shown that the pathogenic defect of osteopetrosis may be intrinsic either to the osteoclast lineage or to the osteoblasts/stromal cells that constitute the bone marrow microenvironment supporting the development and activation of osteoclasts. It has been proven that the defect in osteoclastogenesis in the absence of c-Fos resides within osteoclast lineage, and c-Fos knockout osteoblasts can fully support osteoclast formation both *in vivo* and *in vitro* (Grigoriadis et al., 1994). However, the expression of genes that are essential for osteoclast differentiation has never been mapped using *in situ* techniques. It is widely accepted that RANKL and M-CSF are the two key factors produced by osteoblast/stromal cells for osteoclastogenesis. M-CSF is important not only for the development of osteoclast, but also for macrophages, stimulating the proliferation and differentiation of bipotential precursors. In the absence of RANKL, M-CSF can stimulate even late-stage $RANK^+$ osteoclast precursors into macrophages (Arai et al., 1999). Since mice lacking M-CSF have a severe deficiency of both osteoclasts and macrophages, the fact that more macrophages are present in c-Fos mutant long bones suggests that the production and function of M-CSF are not affected.

RANKL is essential for osteoclast development and T cell differentiation *in vivo*. RANKL deficient mice developed osteopetrosis with no osteoclasts (Kong et al., 1999). *In vitro*, RANKL is necessary and sufficient to support osteoclast differentiation from haematopoietic precursors in the presence of M-CSF (Quinn et al., 1998a). OPG acts as a decoy receptor for RANKL and inhibits osteoclast differentiation both *in vitro* and *in vivo*, and the ratio of RANKL and OPG levels in the bone

microenvironment is critical for the regulation of osteoclast formation (Hofbauer et al., 2000). The expression of RANKL and OPG was investigated in *c-fos* mutant long bones and *in situ* hybridisation showed that RANKL and OPG were strongly expressed in osteoblasts and prehypertrophic chondrocytes of wild-type (Kartsogiannis et al., 1999) and *c-fos* knockout mice. As mRNA levels do not always correlate with secreted proteins, the *in situ* results were further confirmed by immunohistochemistry. Roundy et al. reported that RANKL transcription was upregulated in bone marrow from osteopetrotic mice, including *op/op*, *c-fos*-/- and *mi* (Roundy et al., 2003), which may represent a compensatory mechanism for osteoclast deficiency.

In conclusion, osteoblasts appear to be normal in *c-fos* knockout long bones. The defects in osteoclast differentiation in the absence of c-Fos are therefore unlikely to be caused by dysfunction of osteoblasts.

### 3.7.2 Overexpression of c-Fos affects both osteoclasts and osteoblasts in transgenic osteosarcomas

In this thesis, I also investigated the effect of overexpression of c-Fos on osteoclast differentiation, since osteoclasts appeared to play an important role in the tumour phenotype in these transgenic mice (see also section 1.7.3.2). The expression of osteoclast- and macrophage- specific genes were investigated in c-Fos-induced osteosarcomas, and the results showed that large amount of osteoclasts and macrophages were present in the active bone remodelling area within osteosarcomas, and surprisingly, osteoclastic cells were also present in fibroblastic areas at the margin of osteosarcomas not containing any bone.

High levels of MMP-9, RANK and cathepsin K expression, which are osteoclastic marker genes, were observed in fibroblastic areas of osteosarcomas suggesting that these cells belong to the osteoclast lineage and are functional. It is well known that MMP-9 plays an important role in tumour invasion and metastasis due to their ability to degrade basement membrane collagens (Himelstein et al., 1994). MMP-9 is not only expressed in tumour cells at the margins adjacent to the tumour-stroma interface, but also stromal cells surrounding tumours (Ueda et al., 1996). The MMP-9 secreting human fibrosarcoma cell line HT1080 also invaded a basement membrane *in vitro*. Transfection of HT1080 genomic DNA into non-invasive cells resulted in se-

cretion of MMP-9 and cell invasion, while non-invasive control DNA transfectants did not secrete MMP-9 (Kubota et al., 1991). Despite the high expression of MMP-9 in different invasive cell types, the expression in c-*fos*-induced osteosarcomas apparently remained confined to osteoclast-like cells, as *in situ* hybridisation and immunostaining of adjacent sections showed that these cells also expressed other osteoclast marker genes, such as *TRAP*, *RANK* and *cathepsin K*.

RANK, the receptor for RANKL, mediates the signalling of RANKL and is critical for osteoclast differentiation. The expression of *RANK*, not only in multinucleated cells adjacent to bone, but also in the fibroblastic areas, further confirmed that these cells are committed to the osteoclast lineage. Cathepsin K is considered to be restricted to osteoclasts and osteoclastic bone resorption, but it has been reported that cathepsin K also plays an important and direct role in the infiltration and growth of tumours. Haeckel et al. reported that *cathepsin K* was strongly expressed in chondromas with enhanced immunoreactions at the infiltrative tumour margins. Osteoclastic giant cells were present between tumour tissues and adjacent bone trabeculae, which expressed high levels of *cathepsin K* (Haeckel et al., 2000). In c-*fos* osteosarcomas, the fibrous regions also expressed *cathepsin K*, further supporting the hypothesis that osteoclast-like cells are present in ectopic areas within the tumours.

It is widely accepted that osteoclasts are normally found only in bone tissues. The fact that cells expressing osteoclastic marker genes are present in fibroblastic areas of the tumour margin in c-Fos-induced osteosarcomas, in areas not adjacent to bone tissue, suggests that there is ectopic formation of bone-resorbing cells in the tumours which may precede the formation of bone forming cells during tumour progress, which is different from the normal physiological condition. The presence of ectopic osteoclast-like cells may not be unique to this model, as there are other reports showing that functional osteoclastic cells are present in different bone-related pathologies. For example, the soft tissue tumour, malignant fibrous histiocytoma (MFH) contains functional osteoclasts within the tumour surrounded by muscle (Flanagan and Chambers, 1989). Recently, Riminucci et al. reported that osteoclasts occurred ectopically in fibrous tissues in fibrous dysplasia (FD), which may be due to overexpression of IL-6 (Riminucci et al., 2003).

Osteoclasts are required not only for bone destruction but previous data from our laboratory has suggested that they were also required for bone tumour growth. Wang et al. reported that osteosarcomas developed in Fos-Jun double transgenic mice contained more osteoclasts than c-Fos transgenic mice, and were more remodelled (Wang et al., 1995). Additionally, transgenic mice overexpressing c-*fos* in osteoclasts using a TRAP promoter develop large bone lesions and tumours, which contained numerous osteoclasts (Beedles et al., 1999). On the other hand, tumour formation was almost absent in *c-fos*-/- mice expressing c-Fos transgene (Wang et al., 1995), which suggests that c-Fos-induced tumorigenesis may be gene dose-dependent, or alternatively, that osteoclasts are necessary for osteosarcoma development. Similarly, *mi* mice lacking osteoclasts were resistant to fibrosarcoma cell (from an osteolytic sarcoma tumour, NCTC clone 2472) induced tumorigenesis (Clohisy and Ramnaraine, 1998). However, these mice developed tumours and osteolysis after the osteoclast deficiency was rescued by bone marrow transplantation (Clohisy and Ramnaraine, 1998). On the other hand, injection of tumour cells into *op/op* mice stimulated osteoclastogenesis and tumorigenesis, which was abolished by OPG treatment (Clohisy et al., 1995; Clohisy et al., 2000). Finally, conditioned medium from *op/op* tumour explants contained M-CSF, suggesting that tumour cells secreted the factor that *op/op* lacked and rescued the defect of osteoclast deficiency which then led to tumour formation (Clohisy et al., 1995). Taken together, bone resorbing cells are indispensable for bone tumour development.

It has been well established that macrophages and osteoclasts are derived from same bipotential monocyte/macrophage progenitors, and *in vitro* studies showed that mature macrophages and macrophage cell lines are able to differentiate into osteoclasts in the presence of M-CSF and RANKL. Macrophage infiltration is commonly found in inflammatory diseases including primary and metastatic bone tumours, however, the role of macrophages in tumour development and bone resorption is unclear (Quinn et al., 1998b). It has been reported that human mesenchymal tumour-associated macrophages differentiated into functional osteoclasts when cultured with osteoblastic cells or with RANKL and M-CSF (Quinn et al., 1998b; Yang et al., 2002), and the differentiation of these cells into osteoclasts played an important role in osteolytic metastasis of carcinomas. In order to study the role of macrophages in the growth of c-Fos-induced osteosarcomas, macrophage marker genes expression

was investigated. *In situ* hybridisation of *c-fms*, and immunostaining for F4/80, one of the macrophage-specific surface antigens which has been widely used as a mature macrophages marker (Hattersley and Chambers, 1989b), showed strong expression in numerous macrophage-like mononuclear cells throughout tumour tissues. These results suggest that a large amount of macrophages are present in c-Fos-induced osteosarcomas, and although their function is not entirely clear, it is possible that they could switch to the osteoclast lineage in the presence of appropriate stimuli (Miyamoto et al., 2001).

Taken together, a large amount of multinucleated and mononuclear cells with osteoclast and macrophage phenotypes are present in c-Fos-induced osteosarcomas, not only in bone area but also in fibroblastic areas of the tumour margin not containing any bone, which may play an important role in tumour progress.

Subsequent experiments addressed the reasons for the large number of osteoclasts in c-Fos transgenic tumours, in particular, in the fibroblastic areas. There are two possible explanations: (1) osteoblasts or tumour cells in transgenic mice have a higher potential to support osteoclast differentiation. (2) Overexpression of c-Fos acts directly on osteoclast precursors promoting them to differentiate into mature osteoclasts by certain mechanisms (see also below). *In situ* hybridisation of osteoblast marker genes and osteoblast-derived genes that are critical for osteoclast differentiation were first performed in tumour tissues. As expected, the two osteoblastic genes, *Cbfa-1* and *osteocalcin* were strongly expressed in transformed osteoblasts along the surface of neoplastic bones. Interestingly, fibroblastic cells at the edge of tumours also expressed high level of *Cbfa-1*, the transcription factor essential for osteoblast lineage, but not *osteocalcin*, the mature osteoblastic marker gene, suggesting that these cells might be immature osteoblasts.

As shown before, the local bone marrow microenvironment is critical for osteoclast development *in vivo*. Osteoblasts/stromal cells produce M-CSF and RANKL, which are indispensable for osteoclastogenesis, in response to osteotropic stimuli. At the same time, these cells also secrete OPG, a negative regulator for osteoclastogenesis, and the ratio of RANKL:OPG determines the generation and function of osteoclasts (Hofbauer et al., 2000). To further investigate whether overexpression of c-Fos affected the ability of transformed osteoblasts and fibroblasts to support

osteoclast differentiation, the expression of RANKL and OPG were investigated at different sites in the osteosarcomas. Besides the expected high expression of these cytokines in osteoblastic cells lining neoplastic bone, high levels of RANKL and OPG expression were also observed in tumour fibroblastic cells, suggesting that these transformed fibroblasts/pre-osteoblasts are capable of supporting osteoclast differentiation, and this may explain the presence of osteoclast-like cells in these regions. Similar work has been done on giant cell tumour (GCT) tissues. GCT, also named osteoclastoma, is a rare primary osteolytic tumour of bone. Unlike most other lytic bone tumors, however, GCT contains osteoclast-like cells within the tumour stroma (Atkins et al., 2000). RT-PCR of fractionated GCT tissues showed that GCT stromal cells expressed high level of *RANKL* and *OPG* mRNA (Atkins et al., 2000; Huang et al., 2000). Other studies have shown that in addition to their induction of *RANKL* expression in bone marrow stromal cells, tumour cells, such as myeloma cells, prostate cancer cells and squamous cell carcinoma cells, directly produced RANKL (Nagai et al., 2000; Sezer et al., 2003; Zhang et al., 2001a), and have the potential to support osteoclast formation *in vitro*. As during bone remodelling, bone formation generally follows bone resorption, in malignant tumours, it appears that other transformed cell types apart from osteoblastic cells can also support osteoclast formation and this may be the cellular mechanism responsible for stimulating osteolysis, as in breast cancer metastases or myeloma, or the bone remodelling seen in primary bone tumours.

Taken together, osteoblastic marker genes and key genes for osteoclastogenesis are strongly expressed not only by tumour osteoblasts but also by transformed fibroblasts and/or pre-osteoblasts, which may account for the ectopic osteoclastic cells present in fibroblastic areas.

### 3.7.3 Altered expression of *fra-1* in c-*fos* knockout and transgenic mice

As mentioned in the Introduction (see section .1.7.4), the c-*fos*-related gene, *fra*-1 is now quite well-established as a c-Fos target gene. Thus, to further investigate the potential role of *fra*-1 in the signalling pathway downstream of *c-fos* in osteoclast and osteoblast differentiation, *fra-1* was analysed in *c-fos* knockout long bones, and c-Fos transgenic osteosarcomas.

Fra-1 is the transcriptional target of c-Fos in osteoclast progenitors, and over-expression of Fra-1 rescued the defect of osteoclastogenesis from *c-fos* knockout spleen cells more efficiently than c-Fos (Fleischmann et al., 2000; Matsuo et al., 2000). Furthermore, RANKL-induced *fra-1* expression in haematopoietic precursors is dependent on the presence of *c-fos* (Matsuo et al., 2000). Here, *in situ* hybridisation analysis demonstrated clearly that *fra-1* is specifically and highly expressed by both osteoblasts and osteoclasts in wild-type bones. While it may have been expected that *fra*-1 is expressed in osteoclasts, this has never been demonstrated by any *in situ* techniques until now. Surprisingly, in c-*fos* knockout bones, numerous mononuclear cells expressed high levels of *fra-1*. These cells could be osteoblasts, which express *fra*-1 and would be found throughout the osteopetrotic bone marrow space, or alternatively, they could represent a population of osteoclast precursors. It is possible that in the absence of *c-fos*, *fra-1* transcription is upregulated by RANKL through another unknown pathway(s) in order to compensate for the deficiency of *c-fos* in osteoclastogenesis. However, the endogenous level is not high enough to rescue the defect of osteoclast differentiation compared with exogenous Fra-1 (Matsuo et al., 2000). In fact, Owens et al. have reported that Fra-1 plays a role in osteoclast differentiation distinct from that of c-Fos. In their studies, infection of bipotential osteoclast/macrophage precursor cell lines with retroviruses expressing Fra-1 but not c-Fos caused a dramatic increase in osteoclast differentiation and function in the presence of stromal cells (Owens et al., 1999). Moreover, *in vivo* loss-of-function and gain-of-function experiments showed that Fra-1 is dispensable for osteoclastogenesis. Fra-1 knockout mice generated by injection of Fra-1-/- ES cells into tetraploid wild type blastocysts have TRAP positive osteoclasts present in the long bones at birth (Schreiber et al., 2000). Moreover, Fra-1 transgenic mice developed osteosclerosis due to enhanced bone formation, while osteoclasts were not affected (Jochum et al., 2000). Thus, *in vivo*, the importance of Fra-1 as "the" target gene that mediates the function of c-Fos is still not established.

Finally, previous data showed that osteoblasts are target cells of c-Fos transgene (Grigoriadis et al., 1993). With respect to osteoclasts, previous reports have generally concluded that differentiated, multinucleated osteoclasts don't express high levels of *c-fos*, or only a small proportion of osteoclasts do (Oyama et al., 1998). However, the expression of endogenous *c-fos* or the c-*fos* transgene in the osteoclastic

lineage hasn't been looked at. *In situ* hybridisation showed the expected high expression of the c-*fos* transgene in osteoblasts and other mononuclear cells within the tumour fibroblastic stroma, as this was observed previously (Grigoriadis et al., 1993). However, additional double staining for TRAP activity and *in situ* hybridisation for exogenous c-*fos* showed that only a few TRAP positive multinucleated cells expressed the transgene, while there were many TRAP positive mononuclear cells that expressed high levels of the transgene, which could represent osteoclast precursors. These data suggest that the c-Fos transgene might regulate the differentiation of osteoclast precursors directly. Furthermore, a high level of *fra*-1 expression was observed in osteoclasts and their mononuclear precursors in the tumours. Taken together, these results imply that in the context of the transgenic osteosarcomas, *c-fos* may control osteoclast development through *fra-1* signalling, whereas in the absence of *c-fos*, alternative signalling pathway(s) may regulate the expression of *fra-1*.

Having mapped the expression patterns of osteoclast and osteoblast marker genes *in situ* in c-Fos knockout and transgenic tissues, I focussed in the next chapters on investigating the role of altered levels of c-Fos on osteoclast differentiation using *in vitro* assays and molecular analyses.

# Chapter 4

## *In vitro* osteoclast differentiation from c-Fos knockout and transgenic precursors

## 4. 1 Introduction

As shown in the previous chapter, M-CSF- and RANKL-responsive cells are present in both c-*fos* mutant mouse strains, the c-*fos* transgenics and, despite the absence of osteoclasts, in the c-*fos* knockouts. To investigate further the possible role of c-Fos using these mutants, *in vitro* studies were performed to determine the osteoclastogenic potential of osteoclast precursors in each model system.

As shown in the previous chapter, M-CSF and RANKL responsive cells are still present in *c-fos* knockout long bones, studies were performed to find out whether these precursors have the potential to form osteoclast under *in vitro* condition. This was done using a stroma-free culture system containing non-adherent M-CSF-dependent bone marrow cells as a source of osteoclast precursors. This might be difficult in the c-*fos* knockout animals which have a much reduced bone marrow space due to the osteopetrosis, however, there are significant numbers of marrow cells within these bones, particularly in younger animals. The reason for doing these experiments was based on the hypothesis that the haematopoietic precursors which will differentiate into osteoclasts, and which are blocked in the absence of c-Fos, are different in the bone marrow versus the spleen, *i.e.* that they may represent the "true" precursors, and that the age of the animal may affect the osteoclast potential. After the publication of the c-*fos* knockout phenotype where it was clearly shown that c-*fos* knockout spleen cells failed to differentiate into osteoclasts (Grigoriadis et al., 1994), all subsequent work by Wagner and co-workers was performed using spleen cell-derived haematopoietic cells as a source of osteoclast precursors (Matsuo et al., 2000). While this was a very informative approach, I will investigate in this chapter, the osteoclast potential of haematopoietic cells derived from c-*fos* knockout bone marrow, and compare it with normal precursors and precursors isolated from c-*fos*-overexpressing mice.

## 4.2 Establishment of a stroma-free cell culture system for osteoclast differentiation

Experiments were performed first to optimise the conditions for osteoclast generation. This was done using bone marrow cells from male CD1 adult mice, which were incubated with M-CSF overnight, and non-adherent cells harvested one day

later and being used as a starting cell population. For plating density studies, M-CSF-dependent precursors were plated at cell densities ranging from $10^2$ to $5x10^5$ cells/6mm well and cultured with 25ng/ml of M-CSF and 5ng/ml of RANKL. The results showed there was an increase in osteoclast formation with increasing cell density, which reached a peak at $5x10^4$ cells/well and decreased at higher densities (Fig.4.1.1). Based on this, all subsequent experiments were performed at a plating density of $5x10^4$ cells/6mm well.

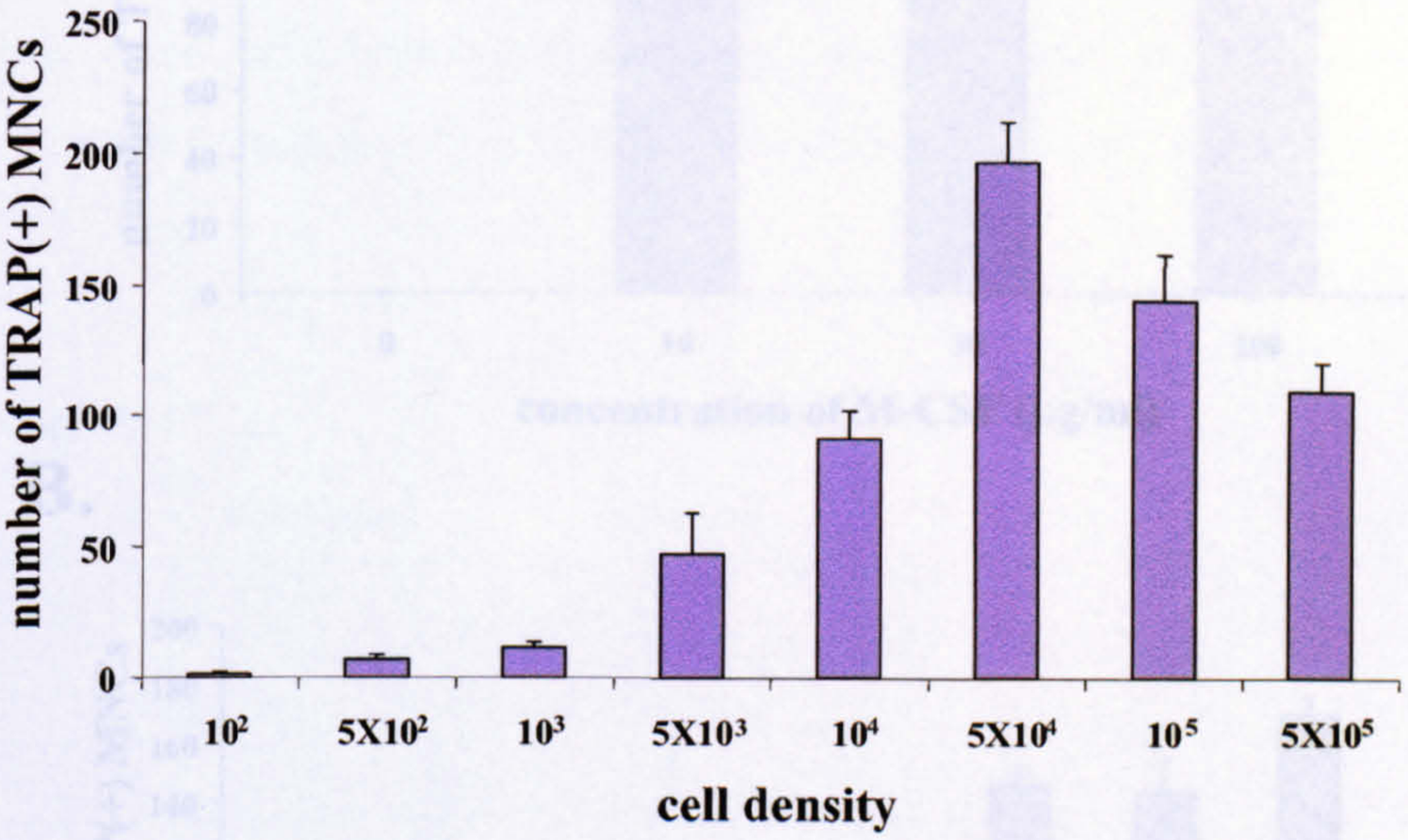
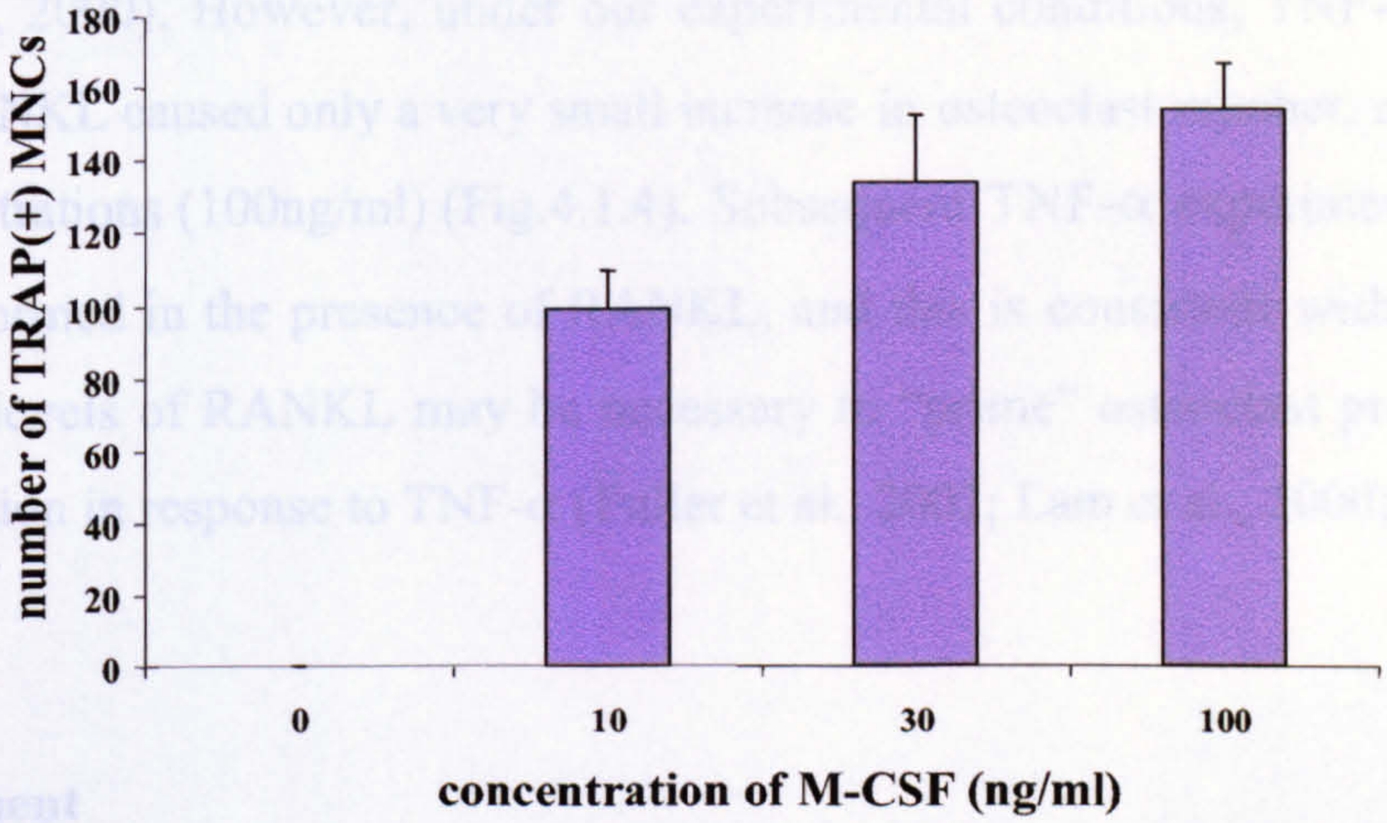



**Fig.4.1.1 The effects of cell density on the formation of TRAP positive multinucleated cells.** M-CSF-dependent bone marrow cells from CD1 mice were plated at different cell densities in 96-well-plates. Cells were cultured with RANKL (5ng/ml) and M-CSF (25ng/ml) for 5 days, then fixed and stained for TRAP activity. The numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment.

I next investigated the dose-dependency of the two major osteoclastogenic cytokines, M-CSF and RANKL. Culture of M-CSF-dependent precursors at a fixed dose of RANKL (25ng/ml) and increasing concentrations of M-CSF showed that M-CSF was essential for osteoclast differentiation, and caused a dose-dependent increase in osteoclast formation (Fig.4.1.2 A). For the RANKL dose-response, M-CSF-dependent cells cultured in a fixed dose of M-CSF (25ng/ml) and different concentrations RANKL also showed that RANKL necessary for inducing osteoclasts and that this response was dose-dependent with concentrations as low as 3ng/ml significantly stimulating osteoclast differentiation (Fig.4.1.2 B). Based on these studies, and unless otherwise stated, all subsequent experiments were performed using

concentrations of M-CSF at 25ng/ml and RANKL at 5ng/ml, for both bone marrow and spleen cultures.

**A.**




**B.**

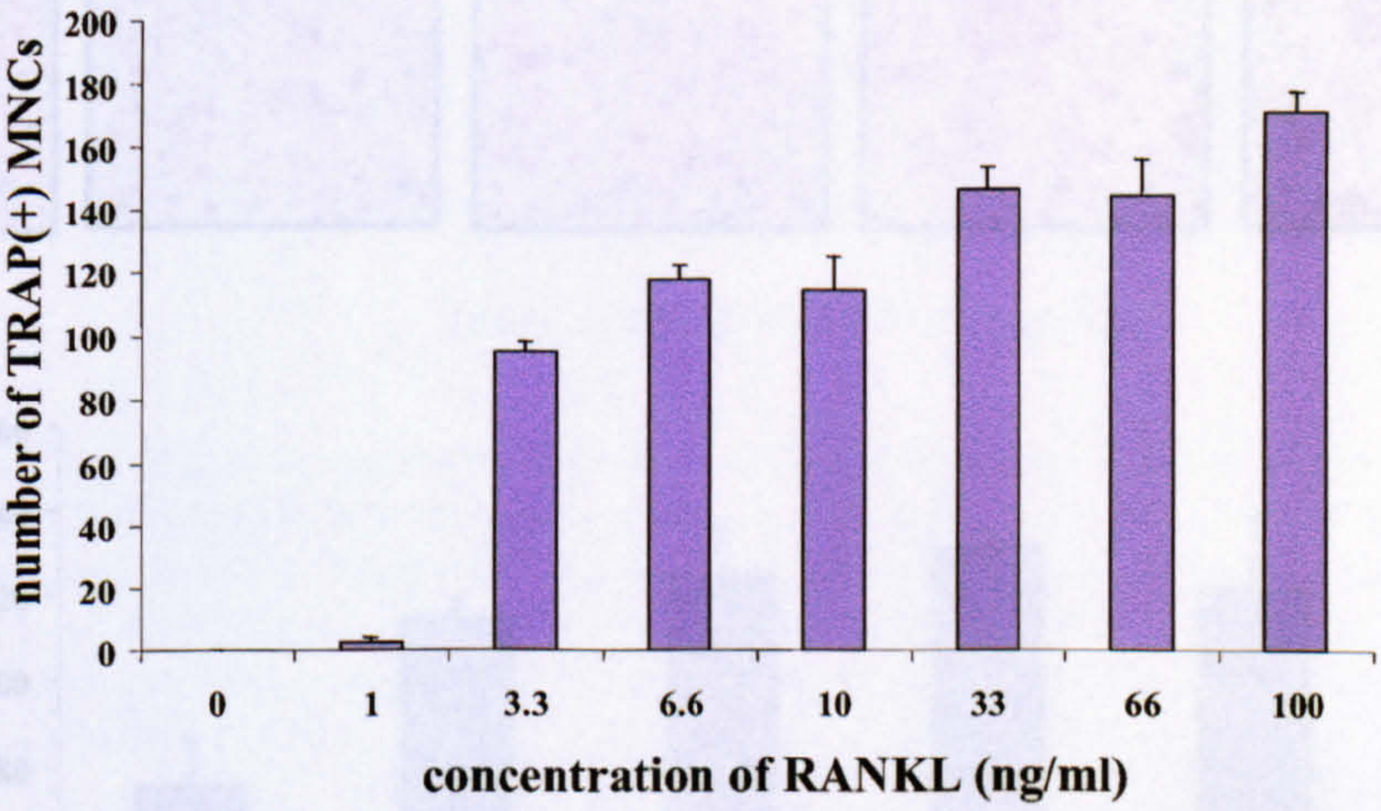



**Fig.4.1.2 M-CSF and RANKL stimulate osteoclast formation in a dose-dependent manner.** Bone marrow cells from CD1 mice were cultured at $5\times10^4$ cells/well in 96-well-plates. Cells were treated with 5ng/ml of RANKL and different concentrations of M-CSF (A), or 25ng/ml of M-CSF and different concentrations of RANKL (B) for 5 days. Cells were then fixed and stained for TRAP activity. The numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment.

Finally, dose-response studies were performed to examine the effects of two additional cytokines which are important for osteoclast differentiation, TGF-β and TNF-α, and which were used in the c-Fos studies. In the presence of M-CSF and

RANKL, TGF-β caused a dose-dependent increase in osteoclast number and size, starting from a concentration as low as 0.01ng/ml (Fig.4.1.3). This is consistent with recently published data (Fuller et al., 2000). With respect to TNF-α, this cytokine has been reported to be able to substitute for RANKL in M-CSF-dependent cultures (Azuma et al., 2000). However, under our experimental conditions, TNF-α in the absence of RANKL caused only a very small increase in osteoclast number, and only at high concentrations (100ng/ml) (Fig.4.1.4). Subsequent TNF-α experiments were therefore performed in the presence of RANKL, and this is consistent with the report that low levels of RANKL may be necessary to "prime" osteoclast precursors for differentiation in response to TNF-α (Fuller et al., 2002; Lam et al., 2000).

**A.**

**TGF-β treatment**


**B.**

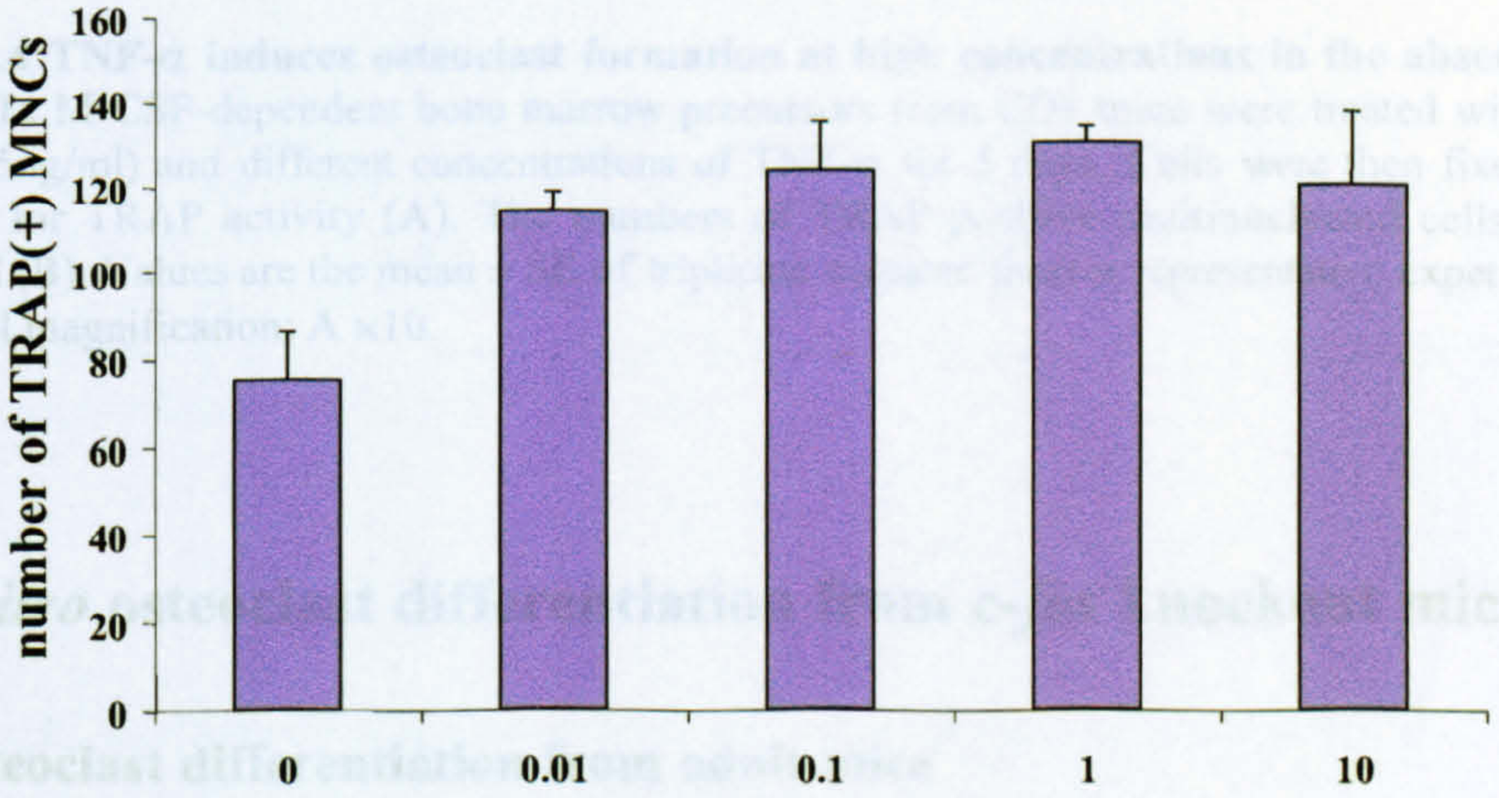



**Fig.4.1.3 TGF-β augments osteoclast formation in a dose-dependent manner in the presence of RANKL and M-CSF.** M-CSF-dependent bone marrow precursors from CD1 mice were treated with RANKL (5ng/ml), M-CSF (25ng/ml) and different concentrations of TGF-β for 5 days. Cells were then fixed and stained for TRAP activity (A). The numbers of TRAP positive multinucleated cells were counted (B). Values are the mean ± SE of triplicate cultures from a representative experiment. Original magnification: A ×10.

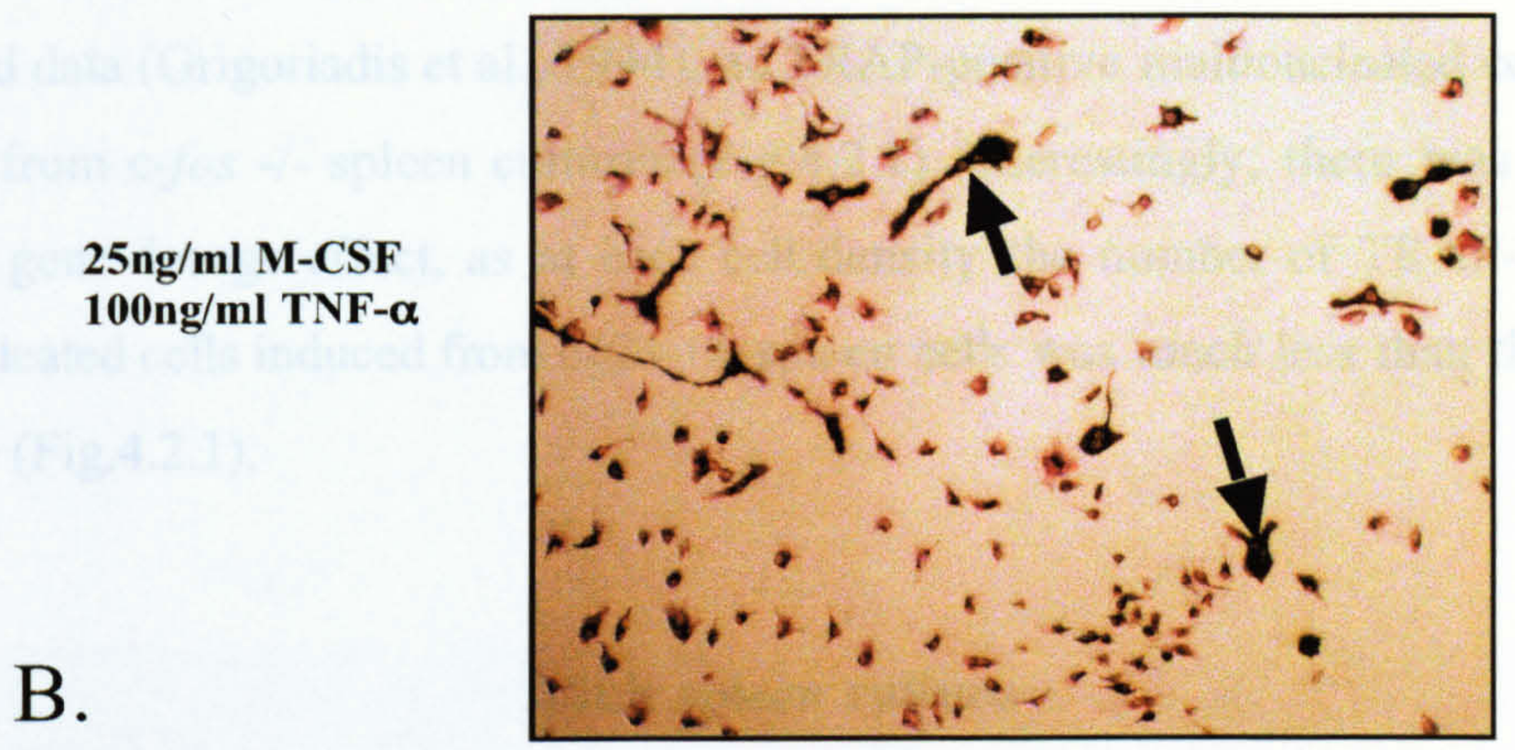



**Fig.4.1.4 TNF-α induces osteoclast formation at high concentrations in the absence of RANKL.** M-CSF-dependent bone marrow precursors from CD1 mice were treated with M-CSF (25ng/ml) and different concentrations of TNF-α for 5 days. Cells were then fixed and stained for TRAP activity (A). The numbers of TRAP positive multinucleated cells were counted (B). Values are the mean ± SE of triplicate cultures from a representative experiment. Original magnification: A ×10.

## 4.3 *In vitro* osteoclast differentiation from c-*fos* knockout mice

### 4.3.1 Osteoclast differentiation from adult mice

I first investigated the differentiation of spleen-derived haematopoietic cells under our experimental conditions. Spleen cells from 2-3 month old *c-fos* +/+, +/-, and -/- male mice were incubated with 25ng/ml of M-CSF overnight. M-CSF-dependent, non-adherent precursors were then plated at different cell densities and cultured with 25ng/ml of M-CSF and 5ng/ml of RANKL for 8 days. The results showed efficient

osteoclast formation which increased with cell density, reaching a peak at $5x10^5$ and $10^6$ cells/well in wild-type cultures, then decreasing dramatically. Consistent with published data (Grigoriadis et al., 1994), no TRAP-positive multinucleated cells were induced from c-*fos* -/- spleen cultures (Fig.4.2.1). Interestingly, there was also an apparent gene-dosage effect, as at each cell density the number of TRAP-positive multinucleated cells induced from c-*fos* +/- spleen cells was much less than that from *c-fos* +/+ (Fig.4.2.1).

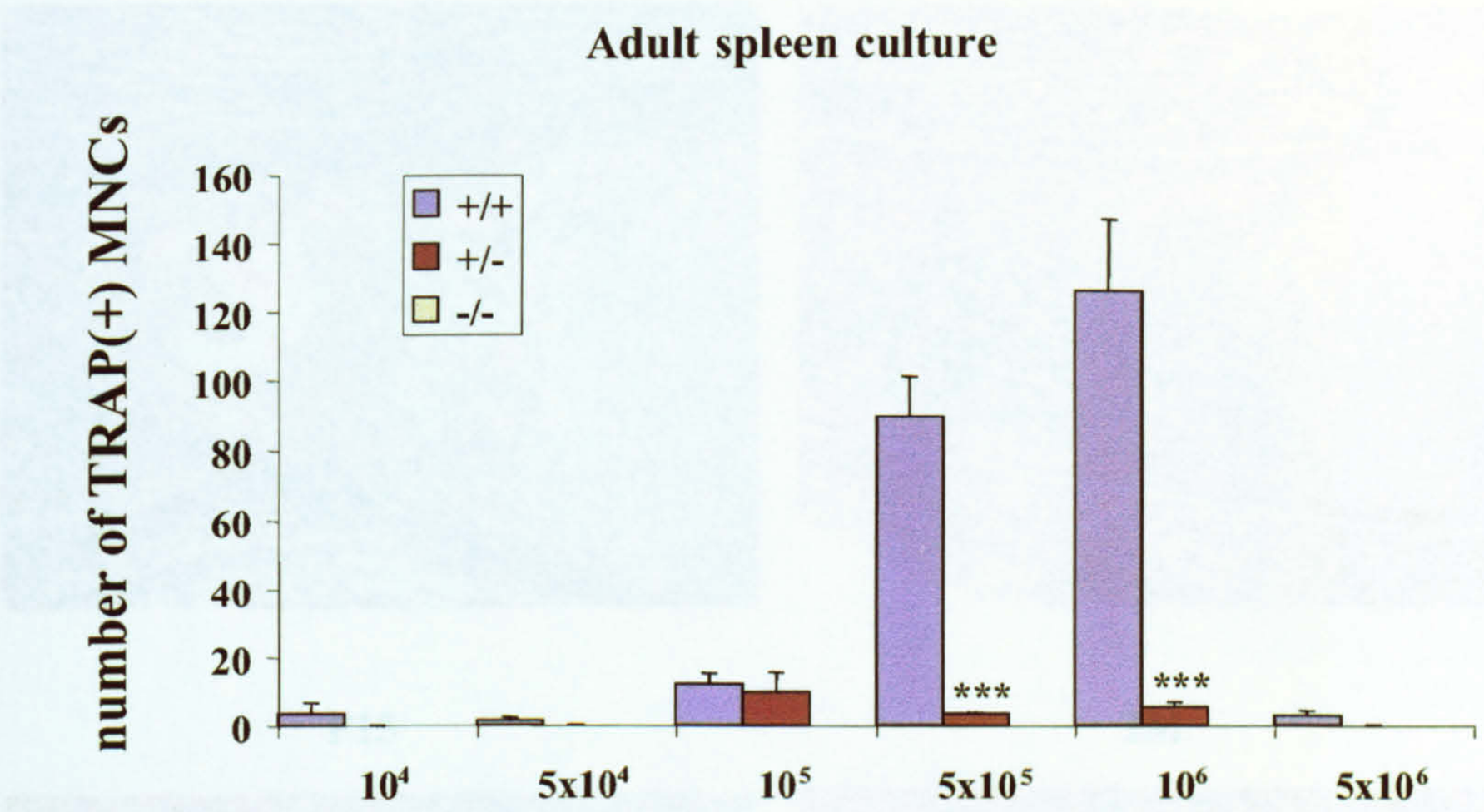



**Fig.4.2.1 TRAP positive multinucleated cells are not induced from *c-fos* -/- adult spleens.** M-CSF-dependent spleen cells from 2-months old male *c-fos* +/+, +/- and -/- mice were plated at different cell densities in 96-well plates. Cells were cultured under standard conditions for 8 days, then fixed and stained for TRAP activity. The numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment. *** $P<0.001$.

I next attempted to isolate cells from bone marrow of adult c-*fos* mutant mice. This was difficult as the osteopetrosis generally worsens with age (Fig.4.2.2), and the number of marrow cells decreases. Nevertheless, enough M-CSF-dependent non-adherent cells could be derived from chopped adult c-*fos* knockout bones to perform an osteoclast differentiation assay. After treatment with RANKL for different days, there were no TRAP-positive cells, either multinucleated or mononuclear, induced from adult *c-fos* mutant bone marrow cultures (Fig.4.2.3 A), whereas +/+ and +/-

cultures showed a time-dependent increase in osteoclast number, starting from day 5-6. Accordingly, there was no resorption pit formation (Fig.4.2.3 B). As also observed with the spleen cultures, the number of TRAP-positive multinucleated cells and resorption pits induced from *c-fos* +/- bone marrow cells at each time point was less than that from *c-fos* +/+ (Fig.4.2.3).

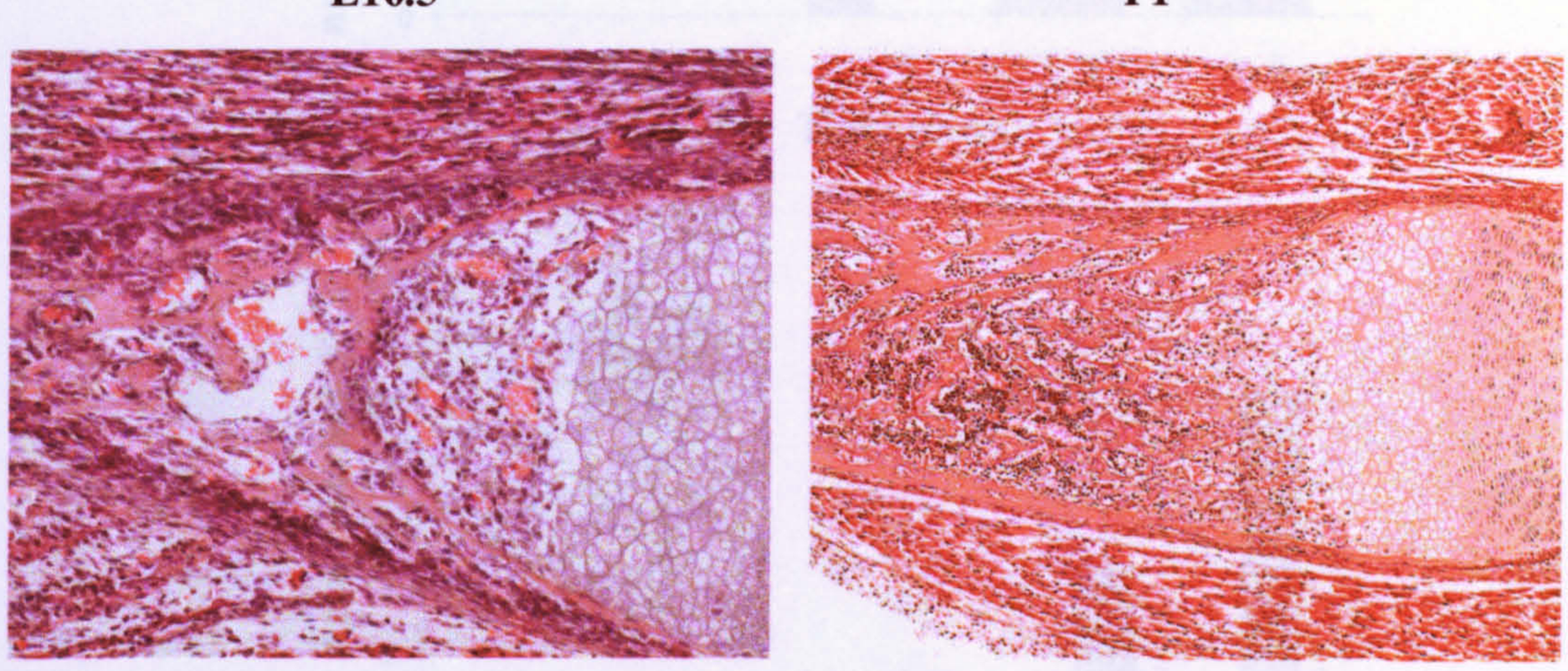



**Fig.4.2.2 Haematoxylin+Eosin staining of long bones from E16.5, postnatal days 1 (P1), 15 (P15) and 2 month old (2M) *c-fos* knockout mice.** The bone marrow space was clearly present in the E16.5 *c-fos* mutant long bone (A), but was gradually reduced in P1 (B) and P15 (C) mutant long bones, and was hardly evident in the adult (2M) mutant long bone (D). Original magnification: A ×20; B-D ×10.

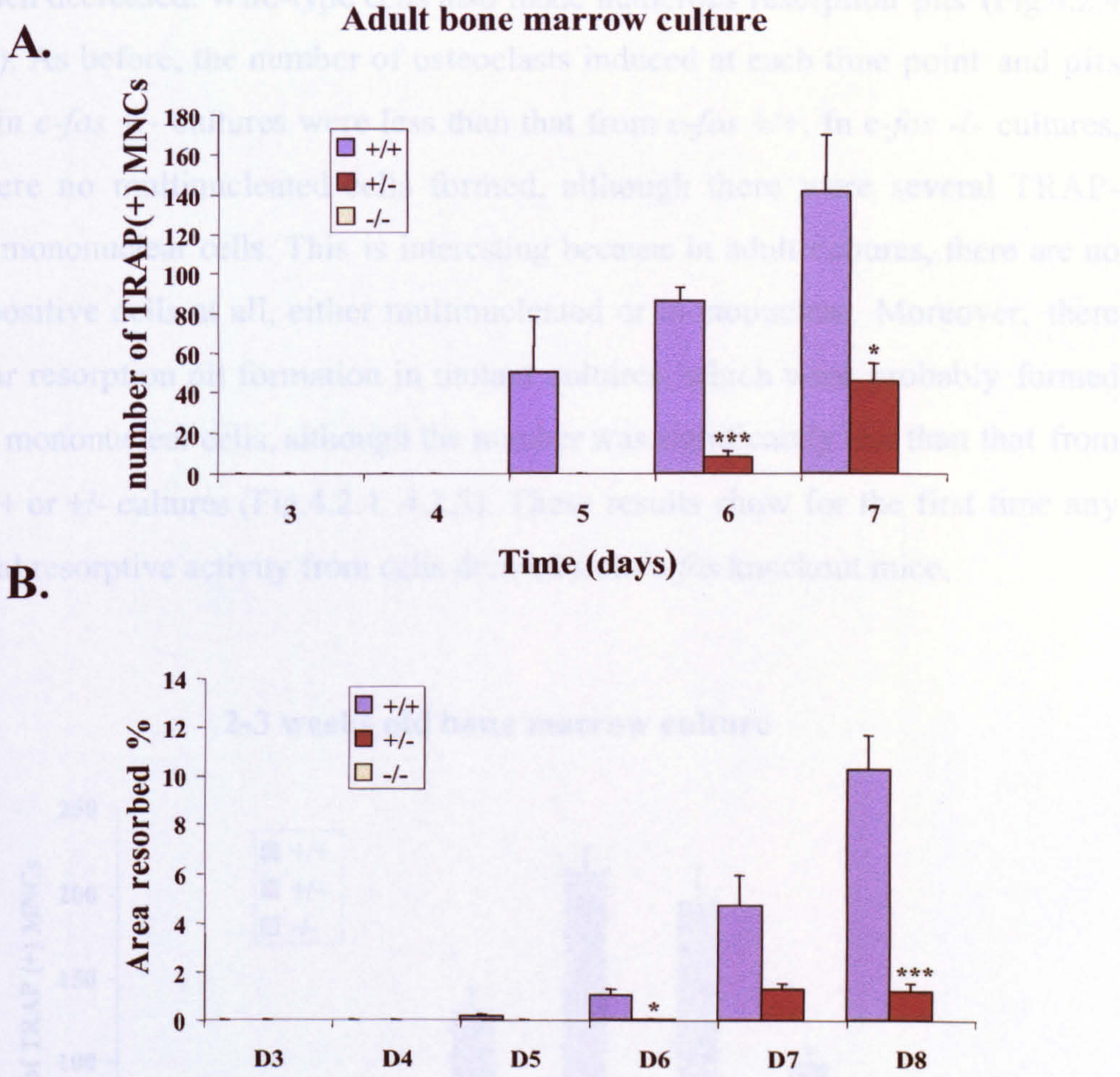



**Fig.4.2.3 TRAP positive cells and resorption pits are not induced from *c-fos* -/- adult bone marrow culture.** M-CSF-dependent bone marrow cells from 2-month old male *c-fos* +/+, +/- and -/- mice were cultured under standard conditions in 96-well plates and on dentine slices. Cells were fixed on different days for analysis of TRAP and resorption. The numbers of TRAP positive multinucleated cells were counted (A), and the percentage of resorbed area on dentine slices was quantified (B). Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; *** $P<0.001$.

## 4.3.2 Osteoclast differentiation from newborn and young mice

To investigate the osteoclast potential of younger mice, where histological evidence showed that the osteopetrosis is not as severe as in adults, osteoclast differentiation assays were performed using cells from young (2-3 week-old) and newborn mice.

Figure 4.2.4 A shows the time course of osteoclast formation from bone marrow precursors of 2-3 week-old mice. In *c-fos* +/+ cultures, there was an increase in the number of TRAP-positive multinucleated cells from day 4 and reached a peak on

day 7, then decreased. Wild-type cells also made numerous resorption pits (Fig.4.2.4 B, 4.2.5). As before, the number of osteoclasts induced at each time point and pits formed in *c-fos* +/- cultures were less than that from *c-fos* +/+. In c-*fos* -/- cultures, there were no multinucleated cells formed, although there were several TRAP-positive mononuclear cells. This is interesting because in adult cultures, there are no TRAP-positive cells at all, either multinucleated or mononuclear. Moreover, there was clear resorption pit formation in mutant cultures, which were probably formed by these mononuclear cells, although the number was significantly less than that from either +/+ or +/- cultures (Fig.4.2.4, 4.2.5). These results show for the first time any functional resorptive activity from cells derived from c-*fos* knockout mice.

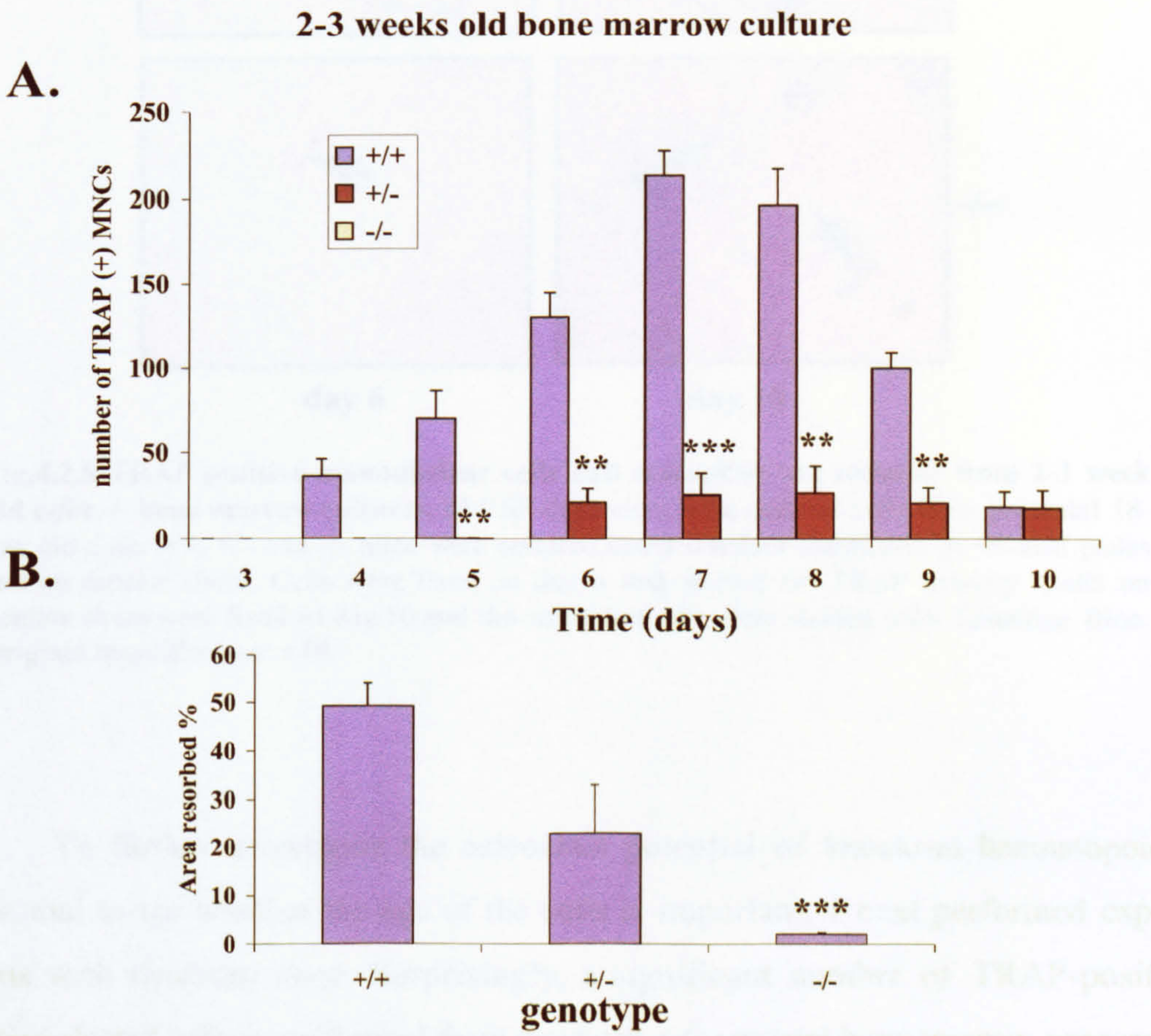



**Fig.4.2.4 Functional TRAP positive mononuclear cells are induced from 2-3 week old *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from postnatal 18-day old *c-fos* +/+, +/- and -/- mice were cultured under standard conditions in 96-well plates and on dentine slices. Cells were fixed on different days, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted (A). Cells on dentine slices were fixed on day 10 and the percentage of resorbed area was quantified (B). Values are the mean ± SE of triplicate cultures from a representative experiment. ** $P<0.01$; *** $P<0.001$.

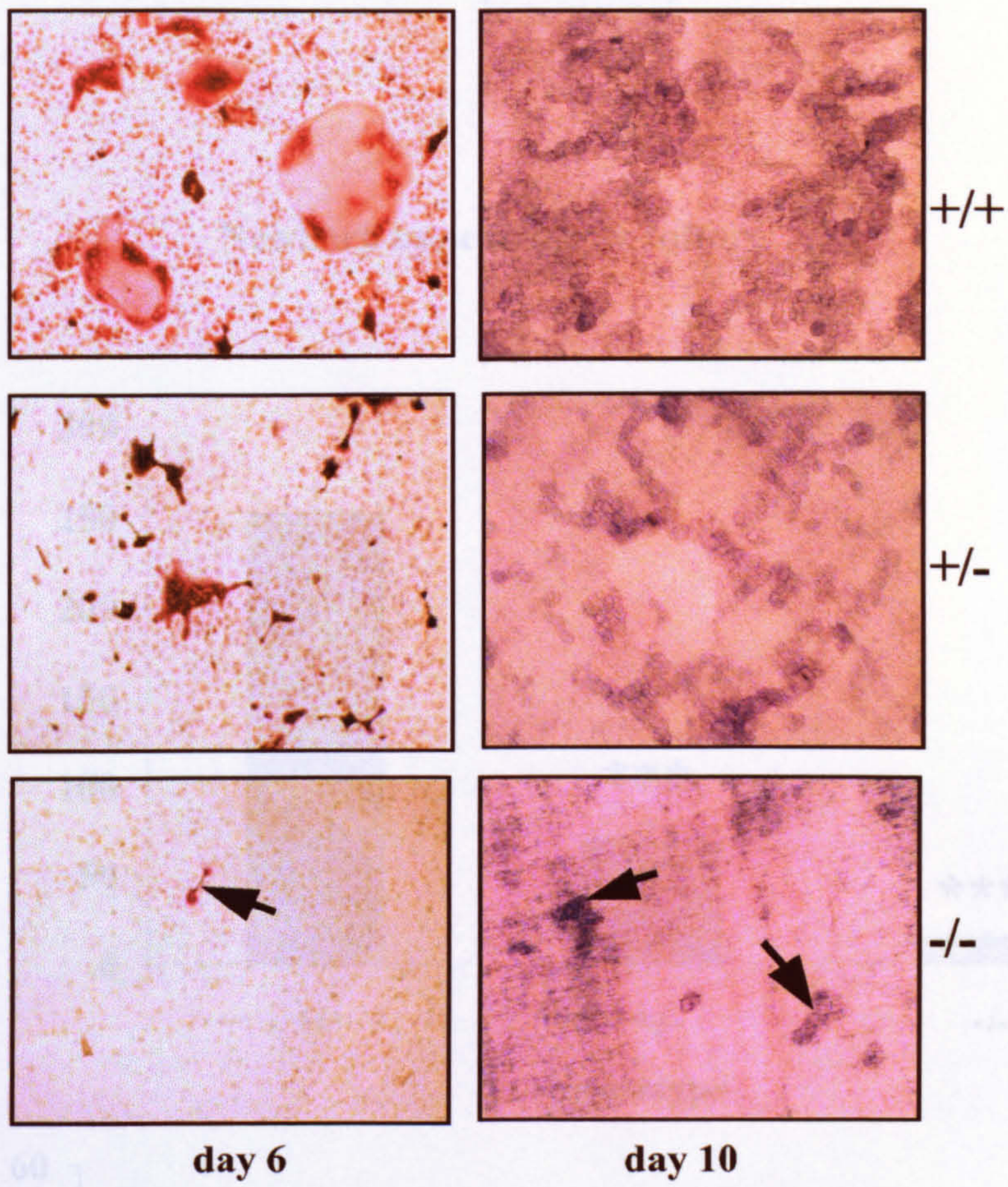



**Fig.4.2.5 TRAP positive mononuclear cells and resorption are induced from 2-3 week old *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from postnatal 18-day old *c-fos* +/+, +/- and -/- mice were cultured under standard conditions in 96-well plates and on dentine slices. Cells were fixed on day 6 and stained for TRAP activity. Cells on dentine slices were fixed on day 10 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

To further investigate the osteoclast potential of knockout haematopoietic cells, and to see whether the age of the cells is important, I next performed experiments with newborn mice. Surprisingly, a significant number of TRAP-positive multinucleated cells were formed from newborn c-*fos* mutant bone marrow precursors (Fig.4.2.6 A, 4.2.7), and these cells formed resorption pits as well (Fig.4.2.6 B, 4.2.7), although the number of TRAP–positive multinucleated cells and pits was much less than that from the wild-type or +/- bone marrow (Fig.4.2.6). This is the

first observation showing the generation of TRAP-positive multinucleated osteoclasts with the ability to make resorption pits from *c-fos* knockout cells.

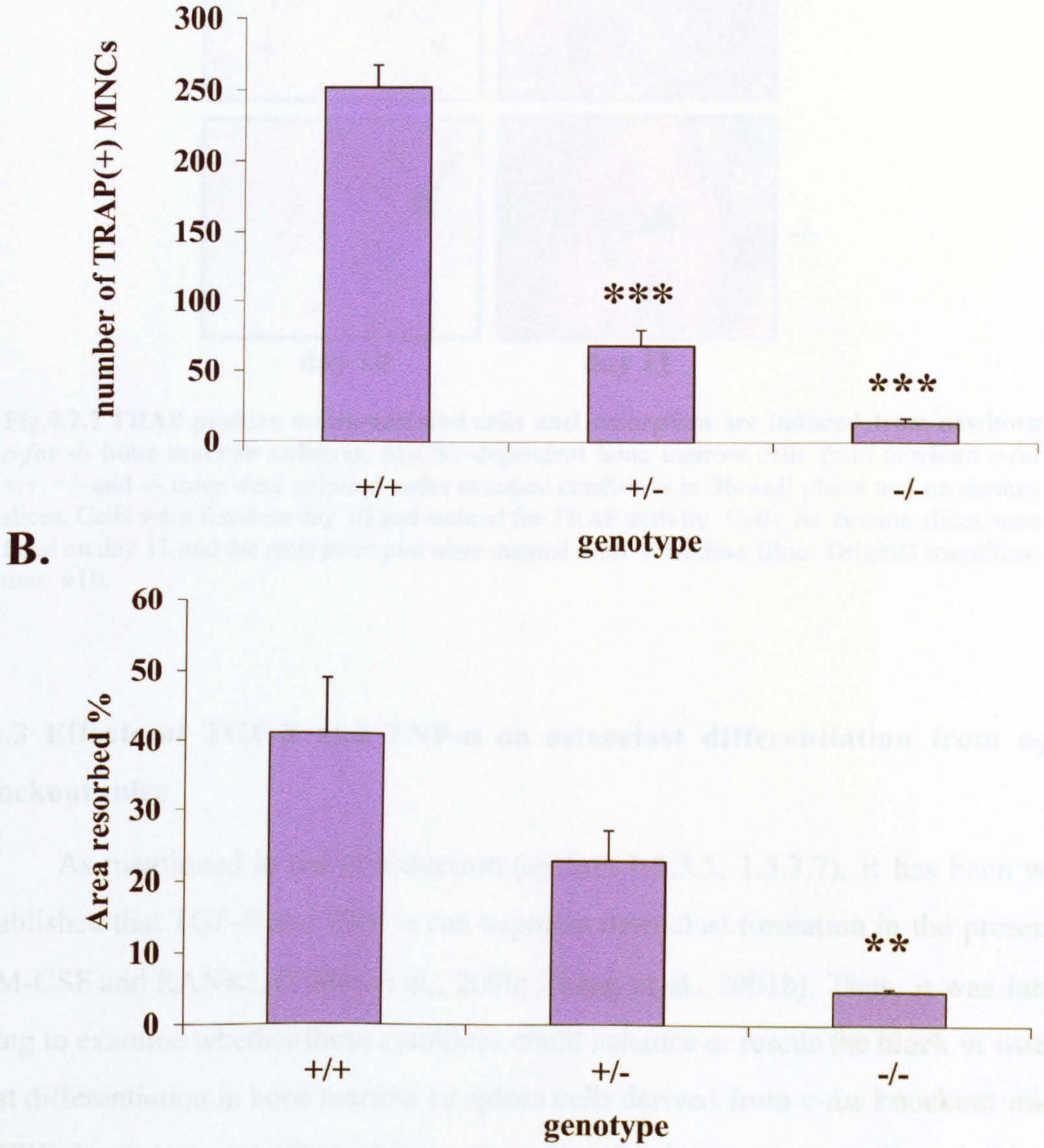



**Fig.4.2.6 Functional TRAP positive multinucleated cells are induced from newborn *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured under standard conditions in 96-well plates and on dentine slices. Cells were fixed on day 10, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted (A). Cells on dentine slices were fixed on day 11 and the percentage of resorbed area was quantified (B). Values are the mean ± SE of triplicate cultures from a representative experiment. ** $P<0.01$; *** $P<0.001$.

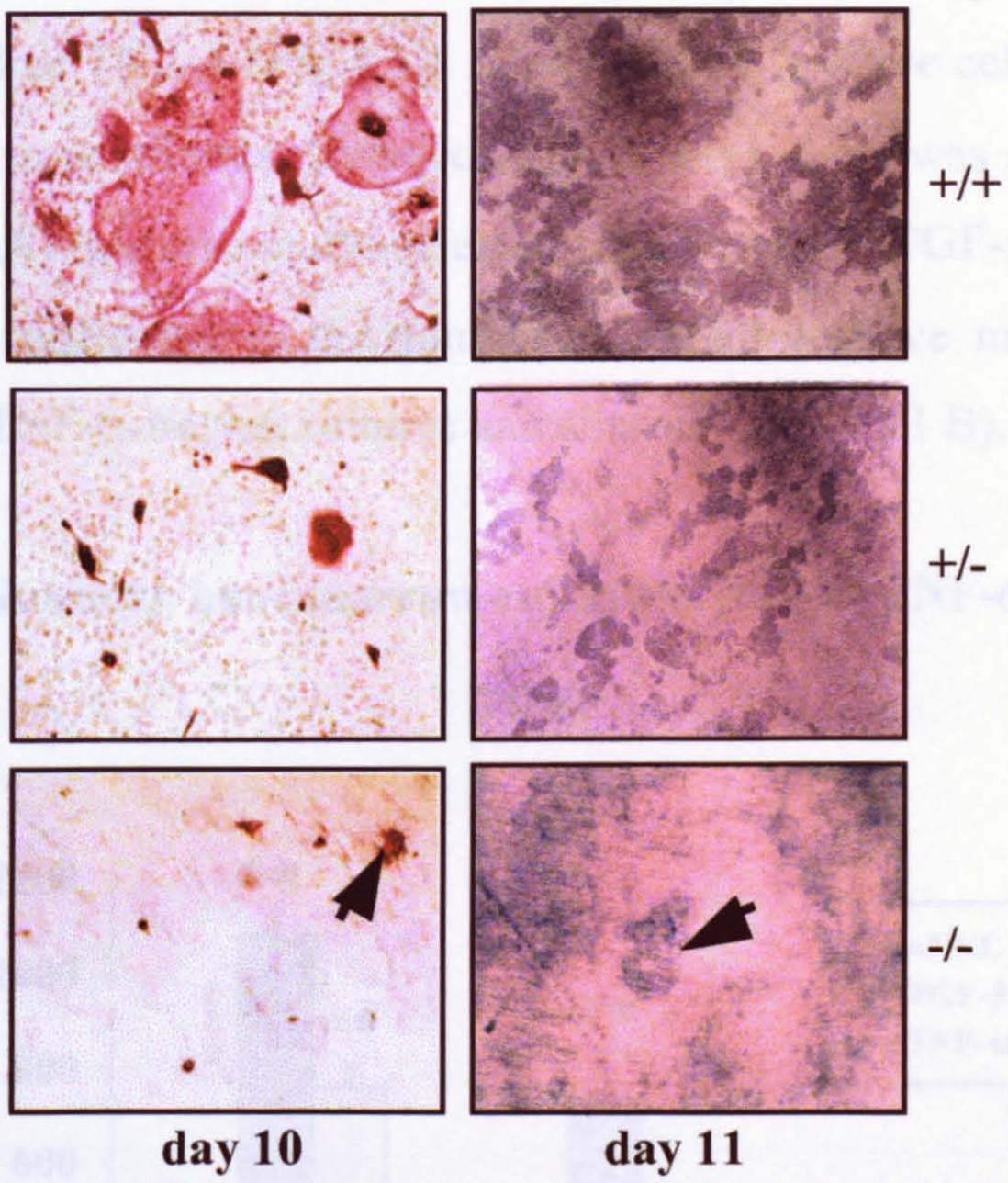



**Fig.4.2.7 TRAP positive multinucleated cells and resorption are induced from newborn *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured under standard conditions in 96-well plates and on dentine slices. Cells were fixed on day 10 and stained for TRAP activity. Cells on dentine slices were fixed on day 11 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

### 4.3.3 Effects of TGF-β and TNF-α on osteoclast differentiation from c-*fos* knockout mice

As mentioned in the Introduction (section 1.5.3.5, 1.5.3.7), it has been well established that TGF-β and TNF-α can augment osteoclast formation in the presence of M-CSF and RANKL (Fuller et al., 2000; Zhang et al., 2001b). Thus, it was interesting to examine whether these cytokines could enhance or rescue the block in osteoclast differentiation in bone marrow or spleen cells derived from c-*fos* knockout mice. M-CSF-dependent non-adherent bone marrow and spleen precursors from newborn, 2-3 week-old and 2-3 month-old c-*fos* +/+, +/- and -/- mice were cultured with TGF-β or TNF-α in the presence of M-CSF and RANKL.

Newborn bone marrow precursors from c-*fos* +/+, +/- and -/- were cultured with TGF-β or TNF-α for 5 or 9 days. On day 5, the number of TRAP-positive

multinucleated cells induced from c-*fos* +/+ and +/- cells were significantly increased by adding TGF-β or TNF-α (Fig.4.3.1 A). No TRAP-positive cells were formed in c-*fos* -/- bone marrow cultures. However, after 9 days, there was still an increase in the number of TRAP-positive multinucleated cells formed in TGF-β-treated cultures, but there was no difference in the number of TRAP-positive multinucleated cells formed between TNF-α-treated cultures and controls (Fig.4.3.1 B).

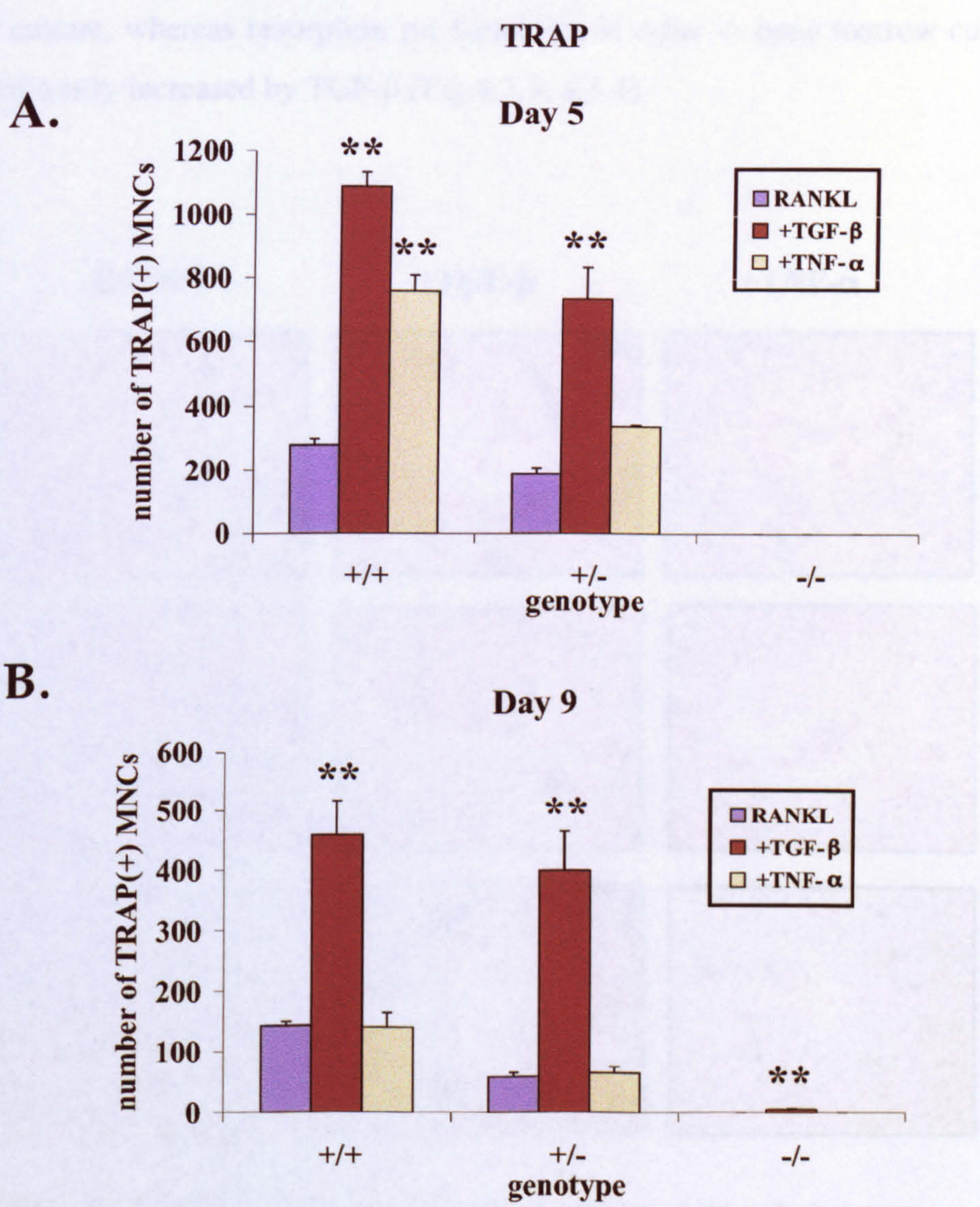



**Fig.4.3.1 TGF-β stimulates TRAP positive multinucleated cell formation from newborn *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (100ng/ml). Cells were fixed on day 5 (A) and day 9 (B), stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment. ** $P<0.01$.

More importantly in day 9 cultures, although TRAP-positive multinucleated cells hadn't formed from c-*fos* -/- cells in control cultures, there was a slight but significant increase in the number of TRAP-positive mononuclear cells and some multinucleated cells induced by addition of TGF-β (Fig.4.3.1 B, 4.3.2). TNF-α failed to stimulate formation of multinucleated cells by day 9 (Fig.4.3.1 B), although many faintly TRAP-positive mononuclear cells were observed (Fig.4.3.2). The resorption assays which were performed in parallel showed that there was no significant increase in pit formation by TGF-β or TNF-α treatment in c-*fos* +/+ or +/- bone marrow culture, whereas resorption pit formation in c-*fos* -/- bone marrow cultures was significantly increased by TGF-β (Fig.4.3.3, 4.3.4).

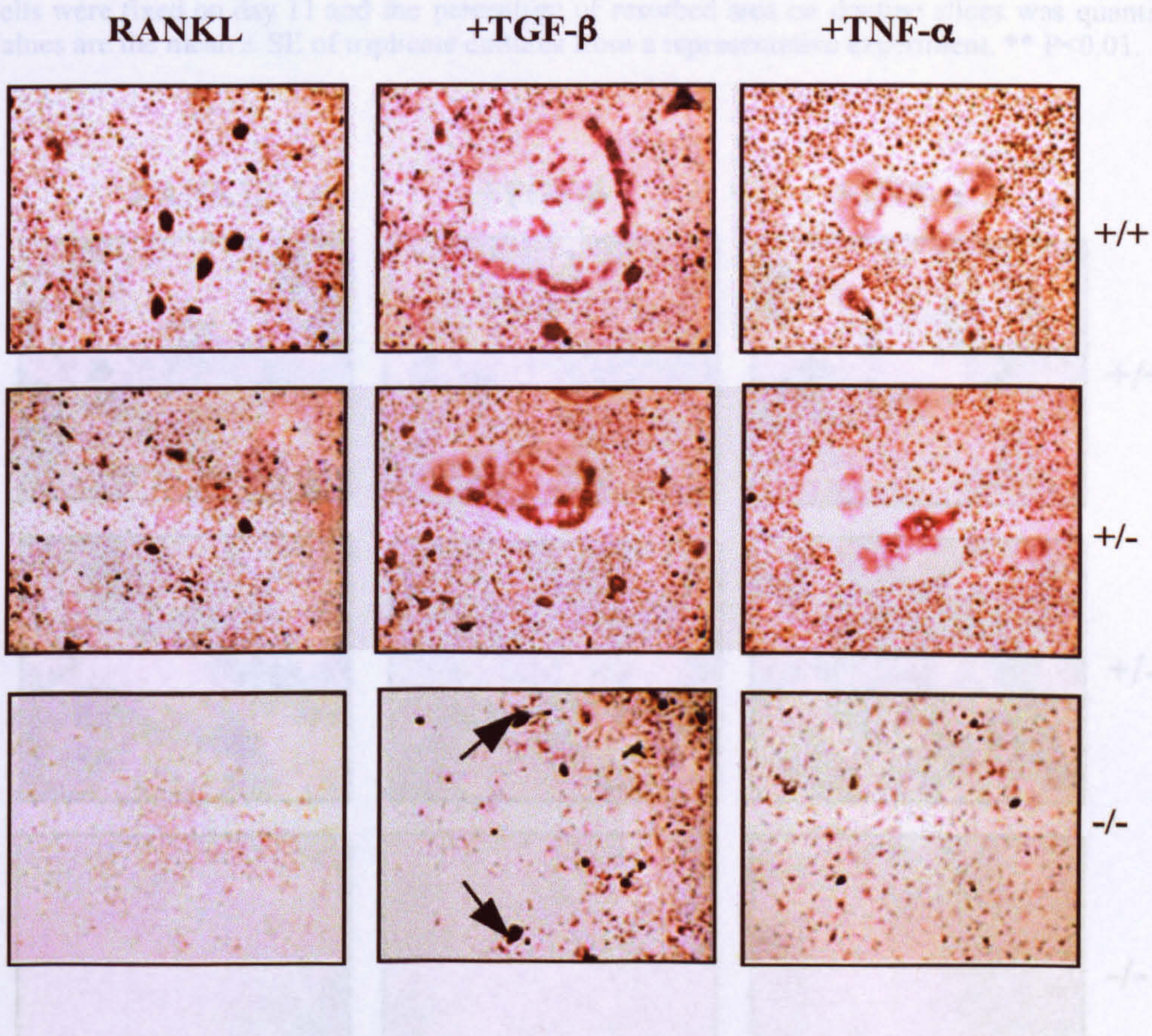



**Fig.4.3.2 TGF-β and TNF-α stimulate TRAP positive multinucleated and mononuclear cell formation from newborn *c-fos* -/- bone marrow cultures, respectively.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (100ng/ml). Cells were fixed on day 9 and stained for TRAP activity. Arrows show TRAP positive multinucleated cells formed in newborn *c-fos* -/- bone marrow cultures treated with TGF-β. Original magnification: ×10.

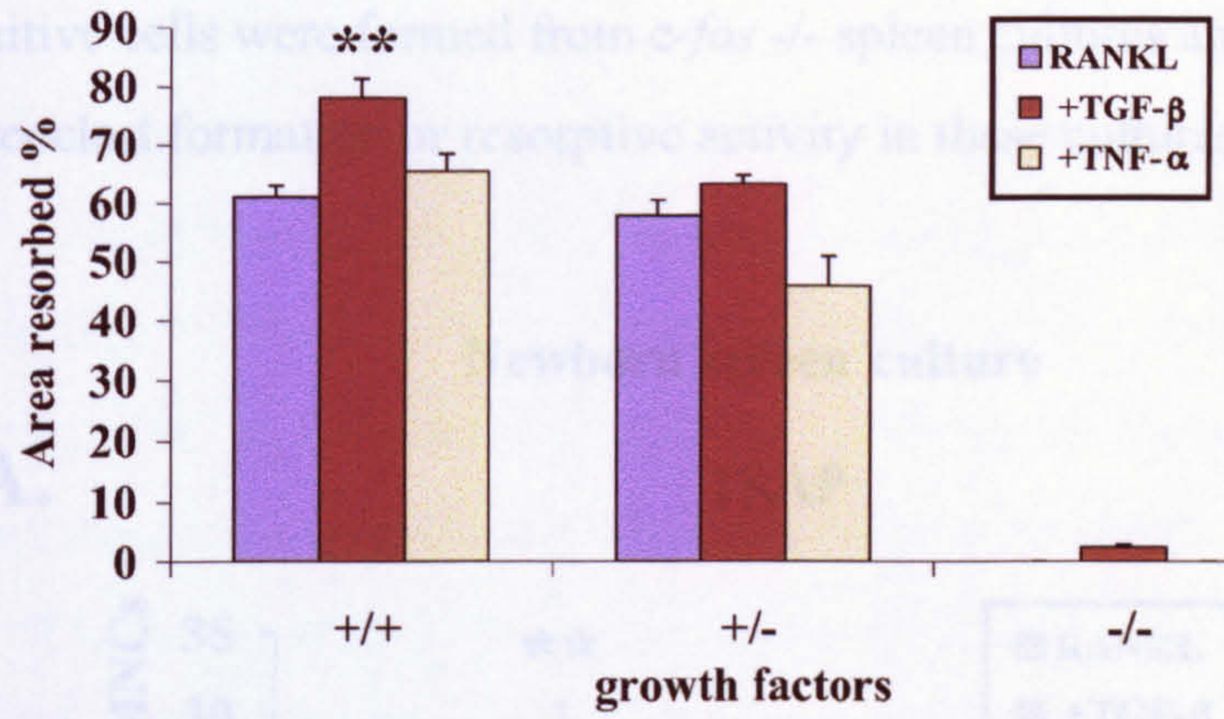



**Fig.4.3.3 TGF-β stimulates resorptive activity of osteoclasts from newborn *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (100ng/ml). Cells were fixed on day 11 and the percentage of resorbed area on dentine slices was quantified. Values are the mean ± SE of triplicate cultures from a representative experiment. ** P<0.01.

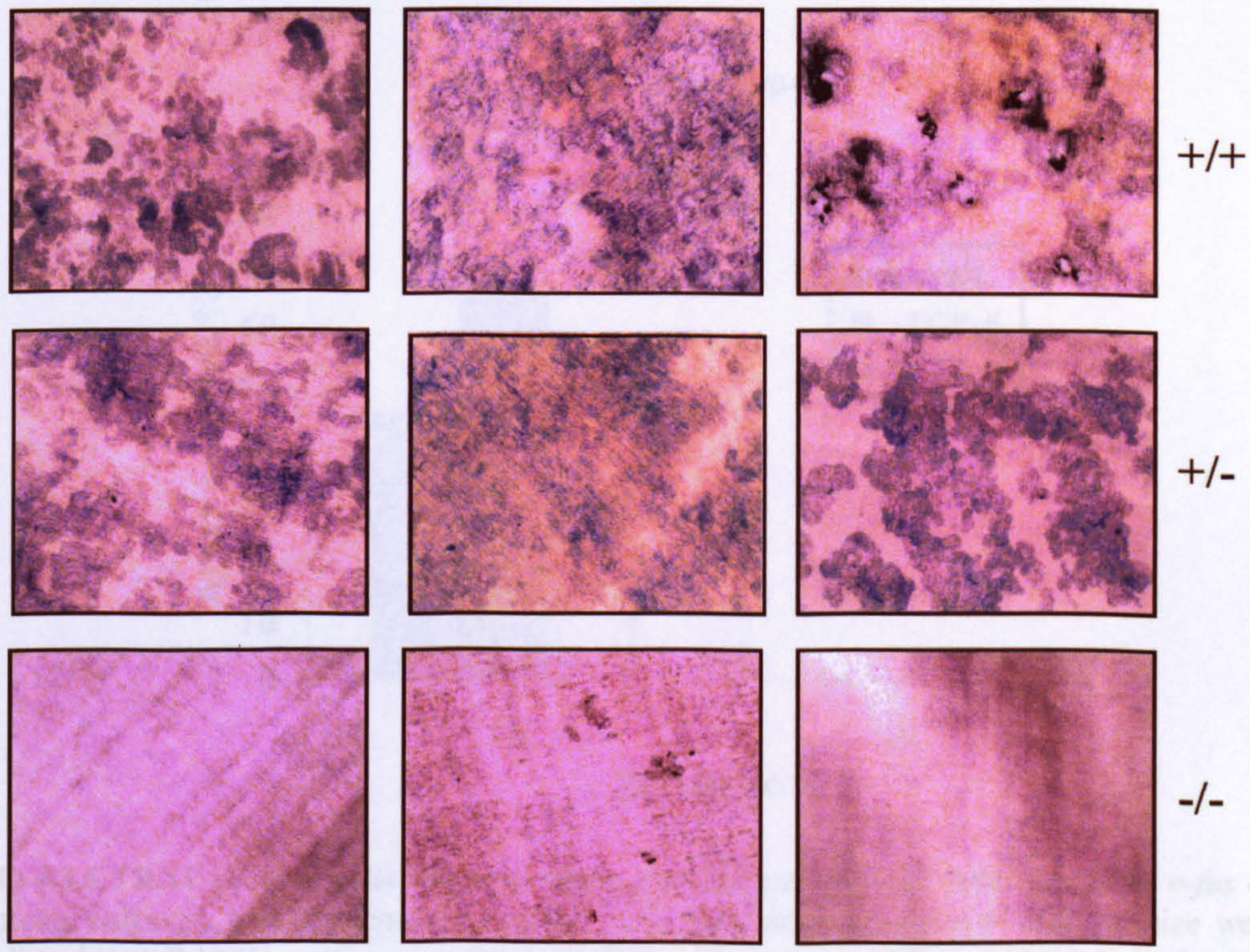



**Fig.4.3.4 TGF-β stimulates resorption in newborn *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from newborn *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (100ng/ml). Cells on dentine slices were fixed on day 11 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

Similar to bone marrow cultures, the number of TRAP-positive multinucleated cells induced from newborn c-*fos* +/- spleen cultures was significantly increased by adding TGF-β. However, in contrast to bone marrow cultures, no TRAP-positive cells were formed from c-*fos* -/- spleen cultures and TGF-β could not restore osteoclast formation or resorptive activity in these cultures (Fig.4.3.5).

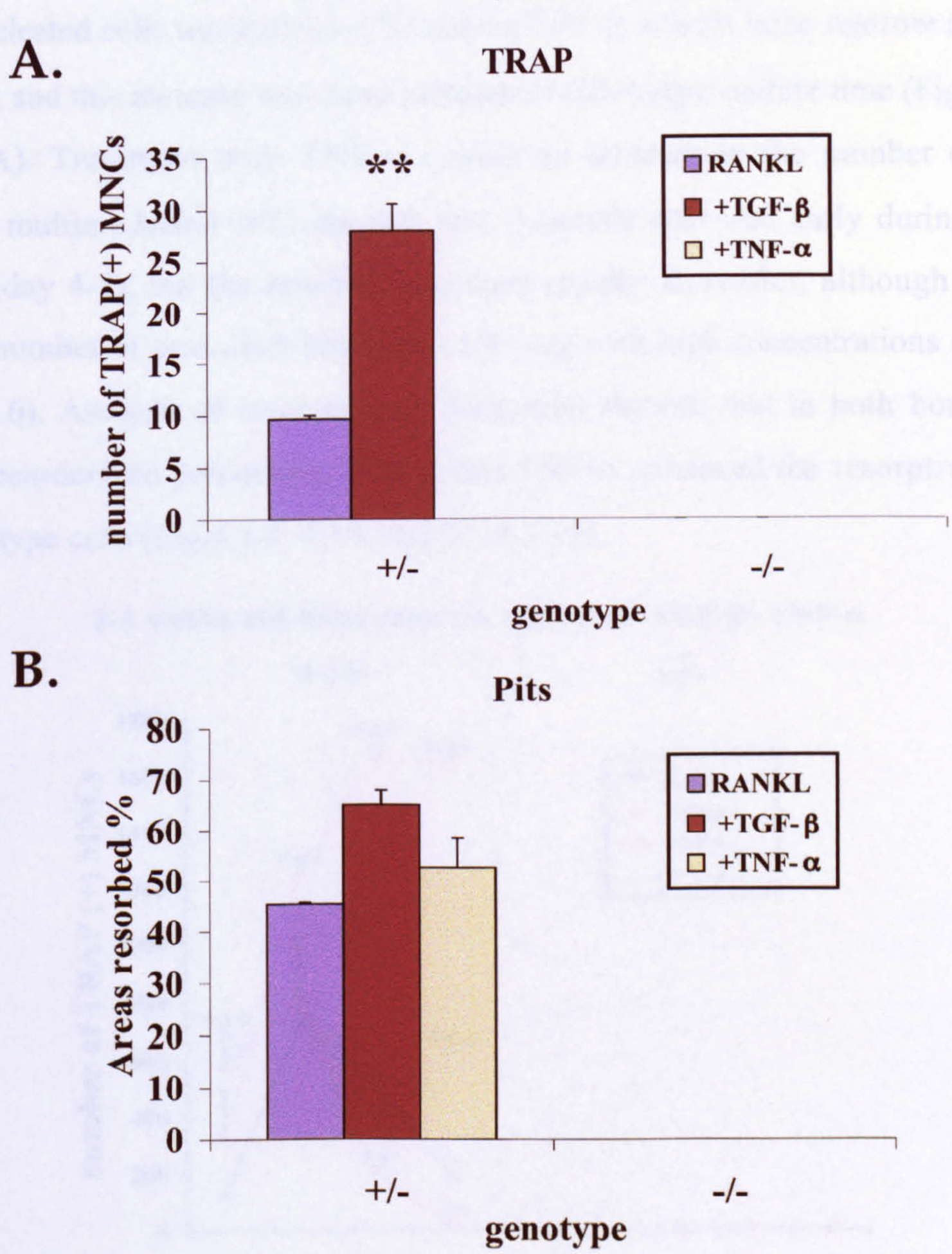



**Fig.4.3.5 TRAP positive cells and resorption pits are not induced from newborn *c-fos* -/- spleen cultures.** M-CSF-dependent spleen cells from newborn *c-fos* +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (100ng/ml) in 96-well plates and on dentine slices. Cells were fixed on day 9, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted (A). Cells on dentine slices were fixed on day 11 and the percentage of resorbed area was quantified (B). Values are the mean ± SE of triplicate cultures from a representative experiment. ** $P<0.01$.

I next investigated the TGF-β and TNF-α effects on the differentiation of bone marrow and spleen cells derived from young and adult mice. Bone marrow and spleen precursors from 2-3 week old c-*fos* +/+ and -/- mice were cultured with TGF-β or TNF-α (10 or 100ng/ml) for 4, 5, 6 and 9 days. Under normal conditions, TRAP-positive multinucleated cells were formed in wild-type bone marrow and spleen cell cultures from day 4, their numbers reached a peak on day 5 and decreased thereafter to day 9 (Fig.4.3.6, 4.3.7 and 4.3.10 A). The number of TRAP-positive multinucleated cells was increased by adding TGF-β in both bone marrow and spleen cultures, and this increase was more prominent with longer culture time (Fig.4.3.6 and 4.3.10 A). Treatment with TNF-α caused an increase in the number of TRAP-positive multinucleated cells and this was generally observed early during the time course (day 4-5), but the number decreased rapidly thereafter, although there was greater number of osteoclast-like cells surviving with high concentrations of TNF-α (Fig.4.3.6). Analysis of resorption pit formation showed that in both bone marrow and spleen-derived precursors, TGF-β and TNF-α enhanced the resorptive activity of wild-type cells (Fig.4.3.8, 4.3.9, 4.3.10, 4.3.11).

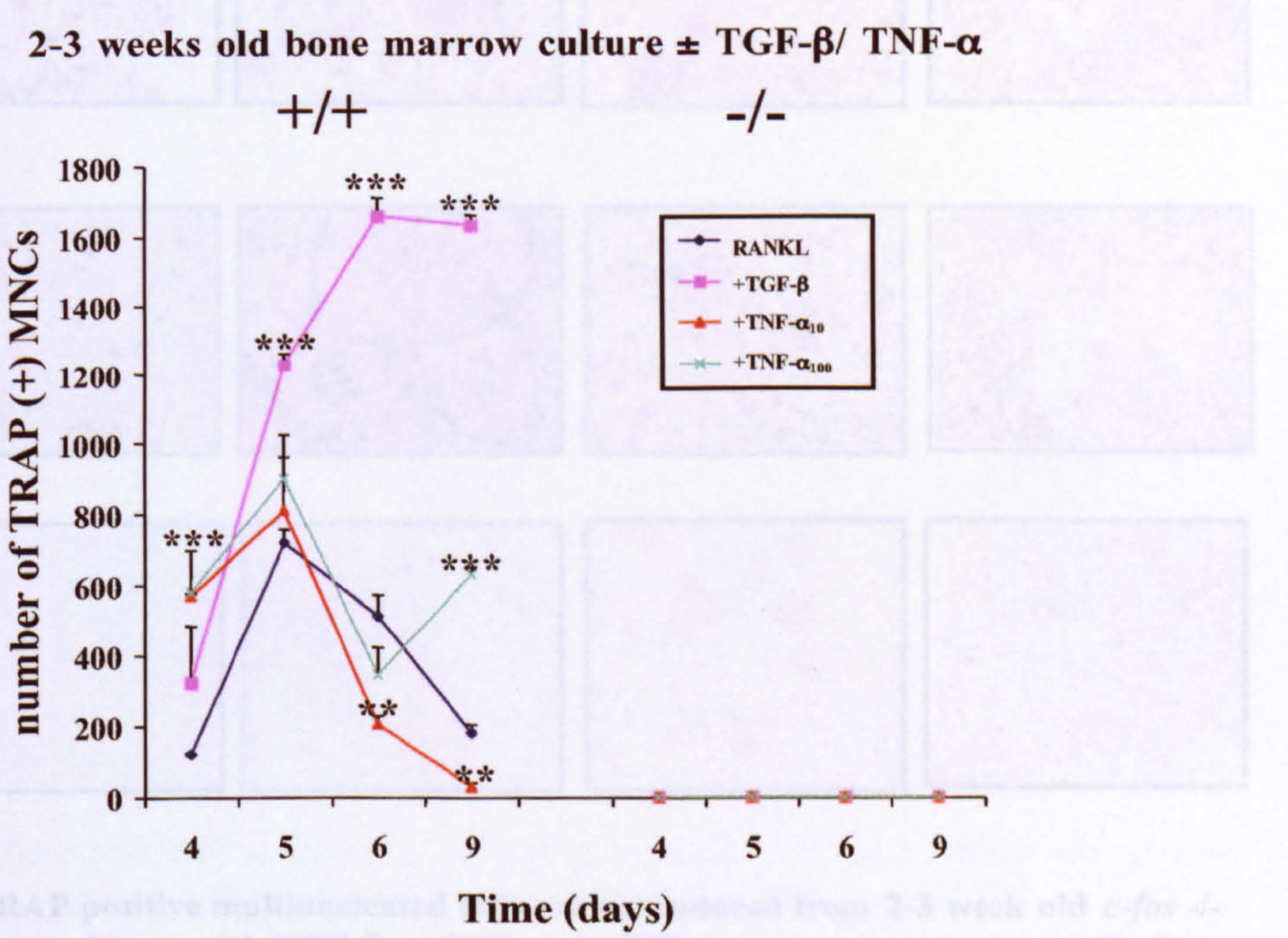



**Fig.4.3.6 TGF-β and TNF-α fail to induce TRAP positive multinucleated cell formation from 2-3 week old *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from postnatal 17-day old *c-fos* +/+ and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on days 4, 5, 6 and 9, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment. ** $P<0.01$; *** $P<0.001$.

With respect to c-*fos* knockout cells, there were, as expected, no TRAP-positive multinucleated cells formed in c-*fos* -/- bone marrow and spleen cultures (Fig.4.3.6). Addition of either TGF-β or TNF-α failed to rescue the formation of TRAP-positive multinucleated cells, although the number of TRAP-positive mononuclear cells was increased (Fig.4.3.7). Analysis of resorption pit formation showed no resorption from spleen cultures (Fig.4.3.10 B), but a few pits formed under control conditions in bone marrow cultures, presumably due to mononuclear osteoclast-like cells (Fig.4.3.8 B, 4.3.9). Addition of TGF-β and to a lesser extent TNF-α, enhanced bone resorption slightly in c-*fos* -/- bone marrow cultures (Fig.4.3.8 B). In contrast, there was no rescue of resorption by either TGF-β or TNF-α in mutant spleen cultures (Fig.4.3.10 B).

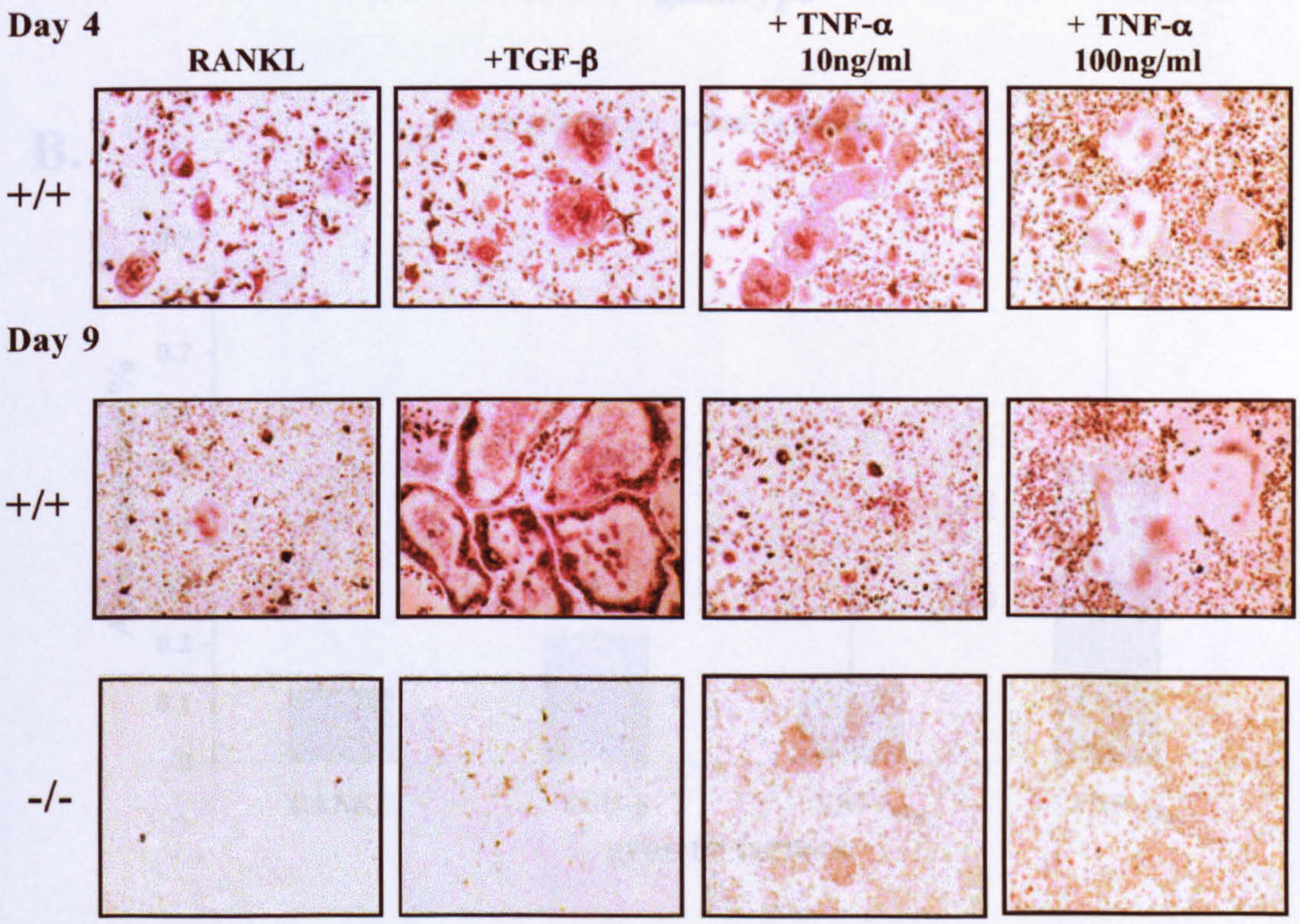



**Fig.4.3.7 TRAP positive multinucleated cells are not induced from 2-3 week old *c-fos* -/- bone marrow cultures with TGF-β or TNF-α.** M-CSF-dependent bone marrow cells from postnatal 17-day old *c-fos* +/+ and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on days 4 and 9, and stained for TRAP activity. Original magnification: ×10.

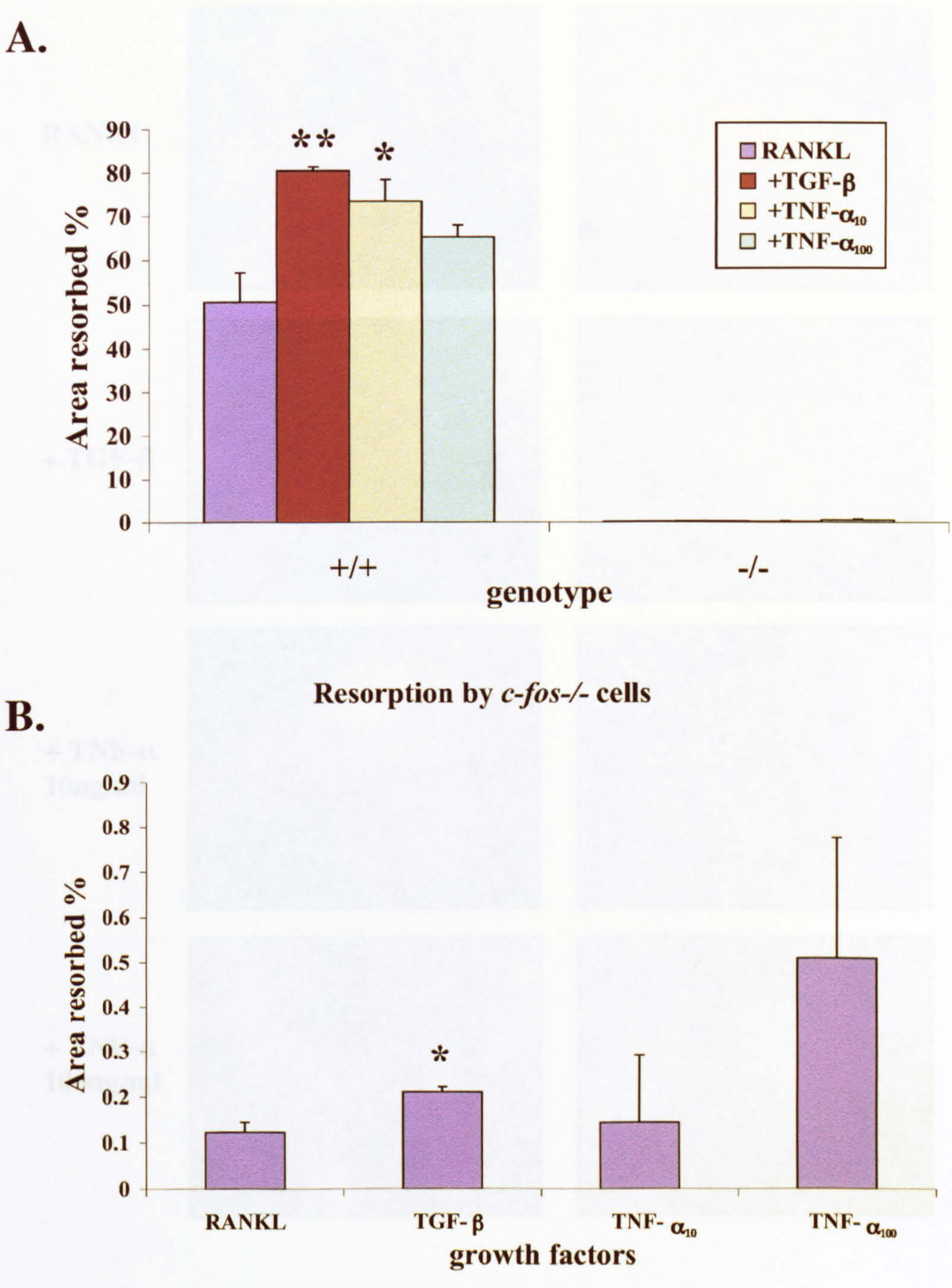



**Fig. 4.3.8 TGF-β and TNF-α enhance the resorptive activity from 2-3 week old *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from postnatal 17-day old *c-fos* +/+ and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on day 11 and the percentage of resorbed area was quantified (A). The data for the *c-fos*-/- bone marrow cultures were replotted on a larger scale in (B). Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; ** $P<0.01$.

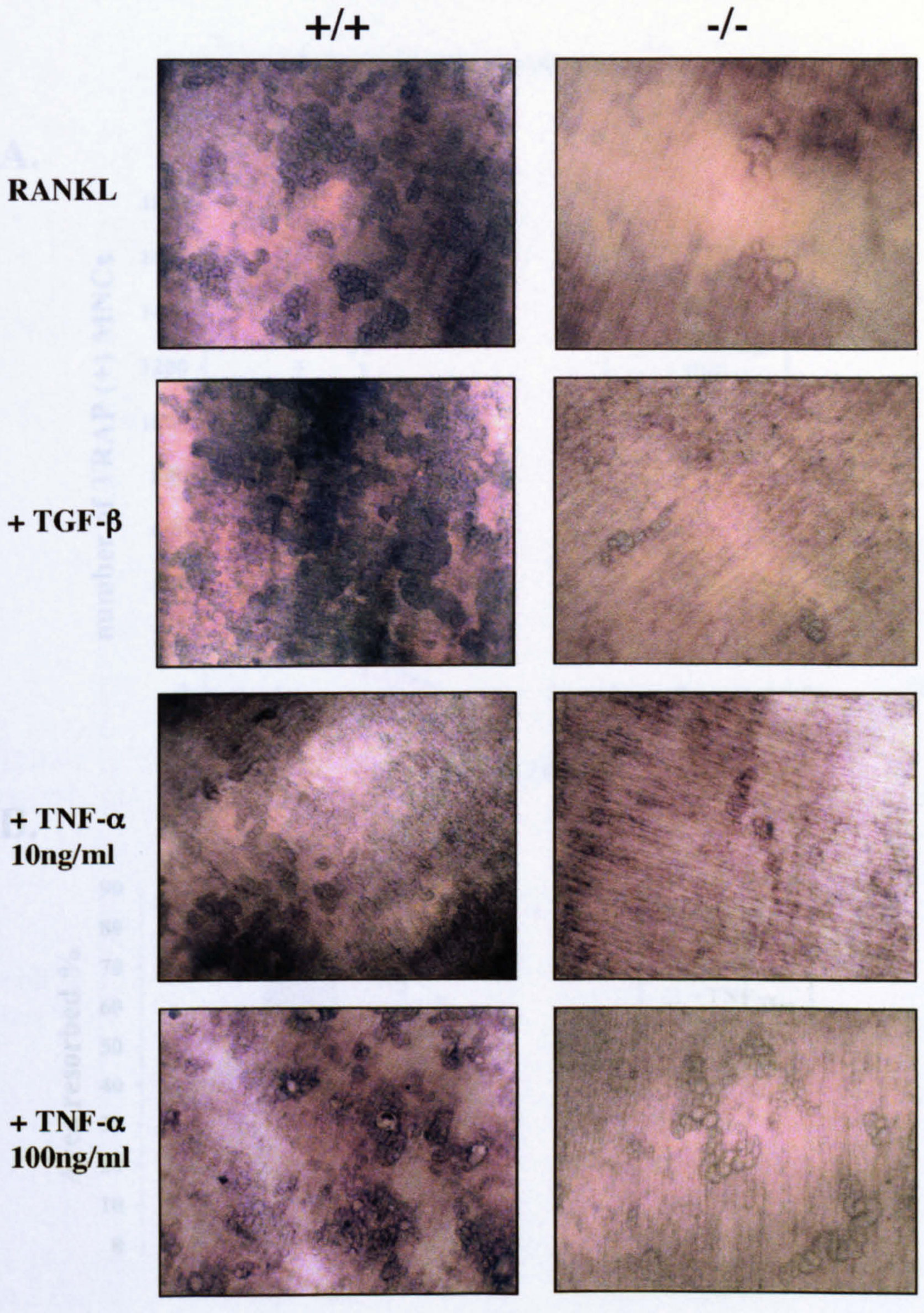



**Fig. 4.3.9 TGF-β and TNF-α enhance osteoclastic resorption from 2-3 week old *c-fos* -/- bone marrow cultures.** M-CSF-dependent bone marrow cells from postnatal 17-day old *c-fos* +/+ and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on day 11 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

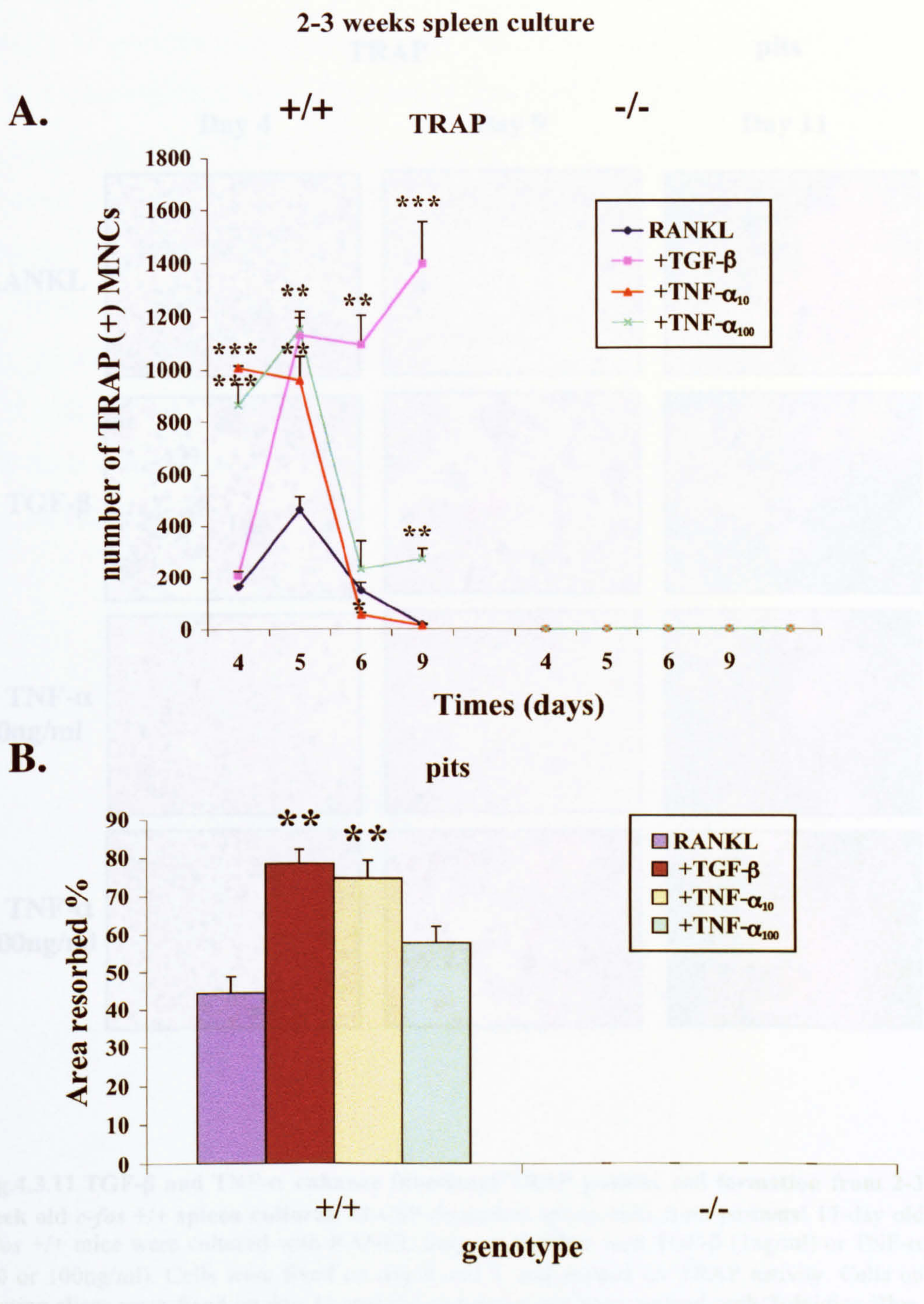



**Fig.4.3.10 TGF-β and TNF-α fail to induce functional TRAP positive cells from 2-3 week old *c-fos* -/- spleen cultures.** M-CSF-dependent spleen cells from postnatal 17-day old *c-fos* +/+ and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on day 4, 5, 6 and 9, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted (A). Cells on dentine slices were fixed on day 11 and the percentage of resorbed area was quantified (B). Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; ** $P<0.001$; *** $P<0.0001$.

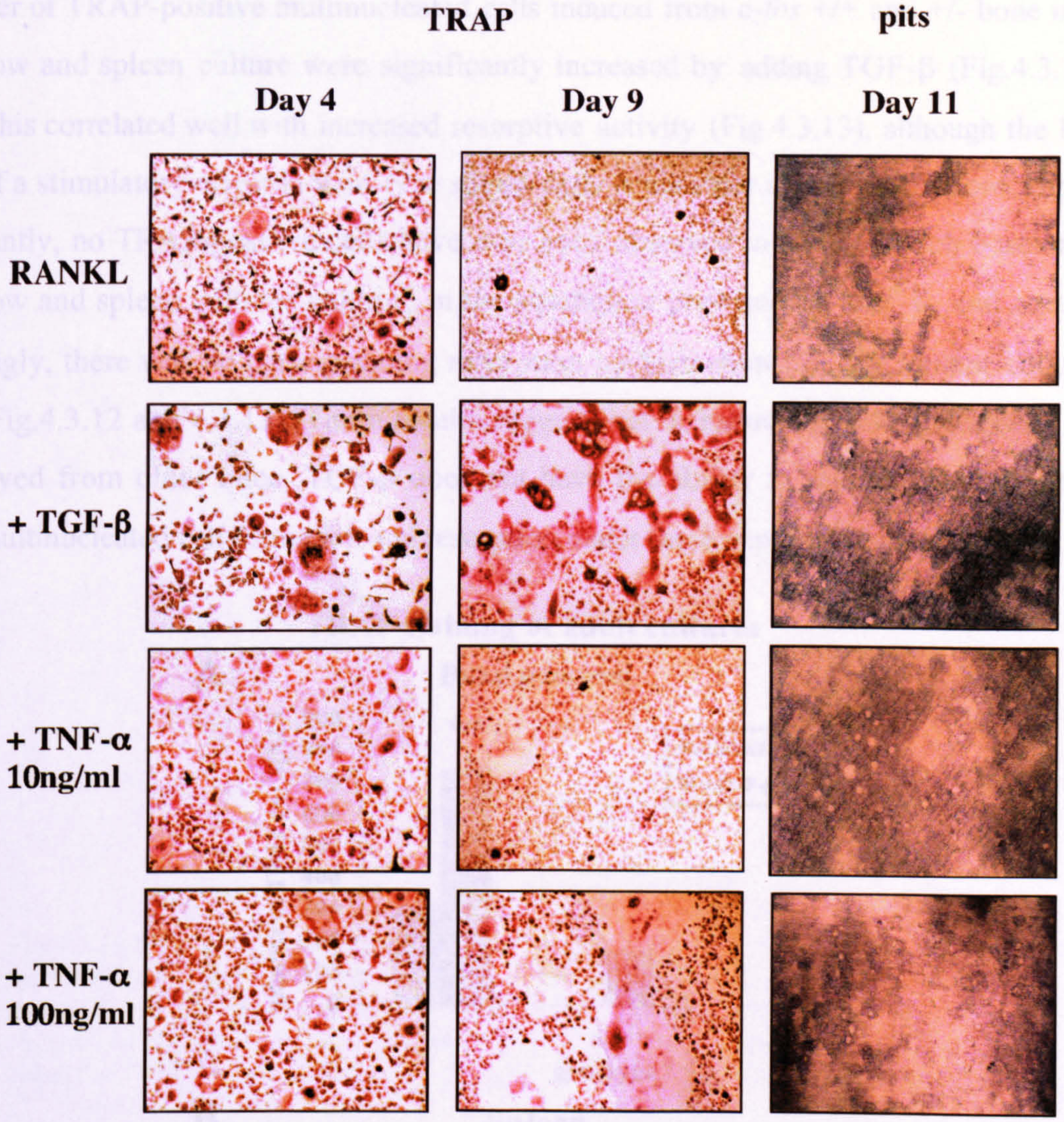



**Fig.4.3.11 TGF-β and TNF-α enhance functional TRAP positive cell formation from 2-3 week old *c-fos* +/+ spleen cultures.** M-CSF-dependent spleen cells from postnatal 17-day old *c-fos* +/+ mice were cultured with RANKL only, or together with TGF-β (1ng/ml) or TNF-α (10 or 100ng/ml). Cells were fixed on day 4 and 9, and stained for TRAP activity. Cells on dentine slices were fixed on day 11 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

Finally, the effects of TGF-β were investigated on bone marrow and spleen cell precursor populations derived from 2-month old c-*fos* knockout mice. The number of TRAP-positive multinucleated cells induced from c-*fos* +/+ and +/- bone marrow and spleen culture were significantly increased by adding TGF-β (Fig.4.3.12). This correlated well with increased resorptive activity (Fig.4.3.13), although the lack of a stimulatory effect in wild-type spleen cells is not clear at this time. More importantly, no TRAP-positive cells have ever been observed in c-*fos* -/- adult bone marrow and spleen cultures cultured in the absence or presence of TGF-β, and accordingly, there was no corresponding resorption pit formation in *c-fos* mutant cultures (Fig.4.3.12 and 4.3.13). These results suggest that in haematopoietic precursors derived from older mice, TGF-β does not have the ability to rescue TRAP-positive multinucleated cell formation and resorption under these experimental conditions.

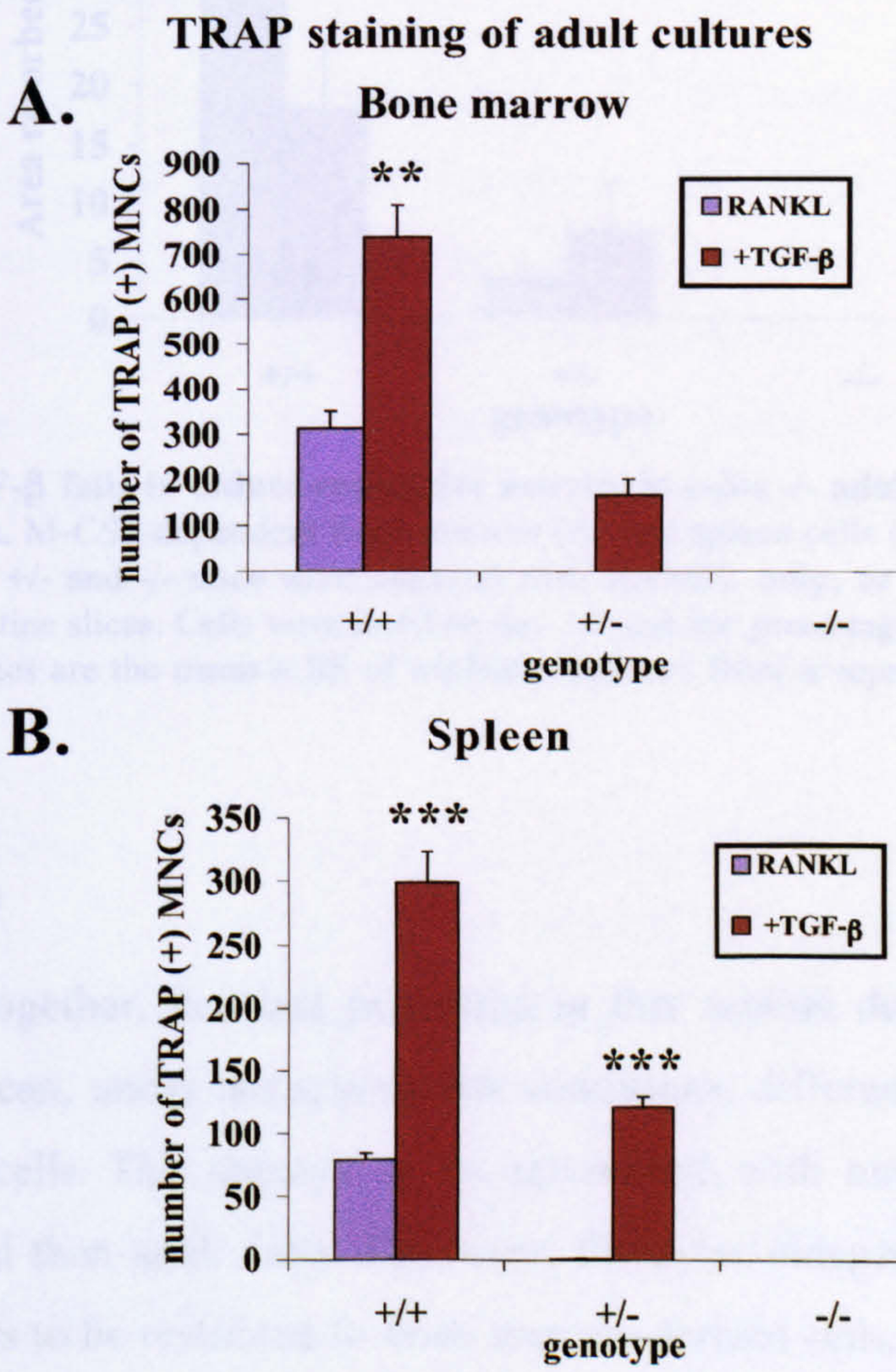



**Fig.4.3.12 TGF-β fails to induce TRAP positive cell formation from *c-fos* -/- adult bone marrow and spleen cultures.** M-CSF-dependent bone marrow (A) and spleen cells (B) from 2-month old male *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml). Cells were fixed on day 9, and stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment. ** P<0.001; *** P<0.0001.

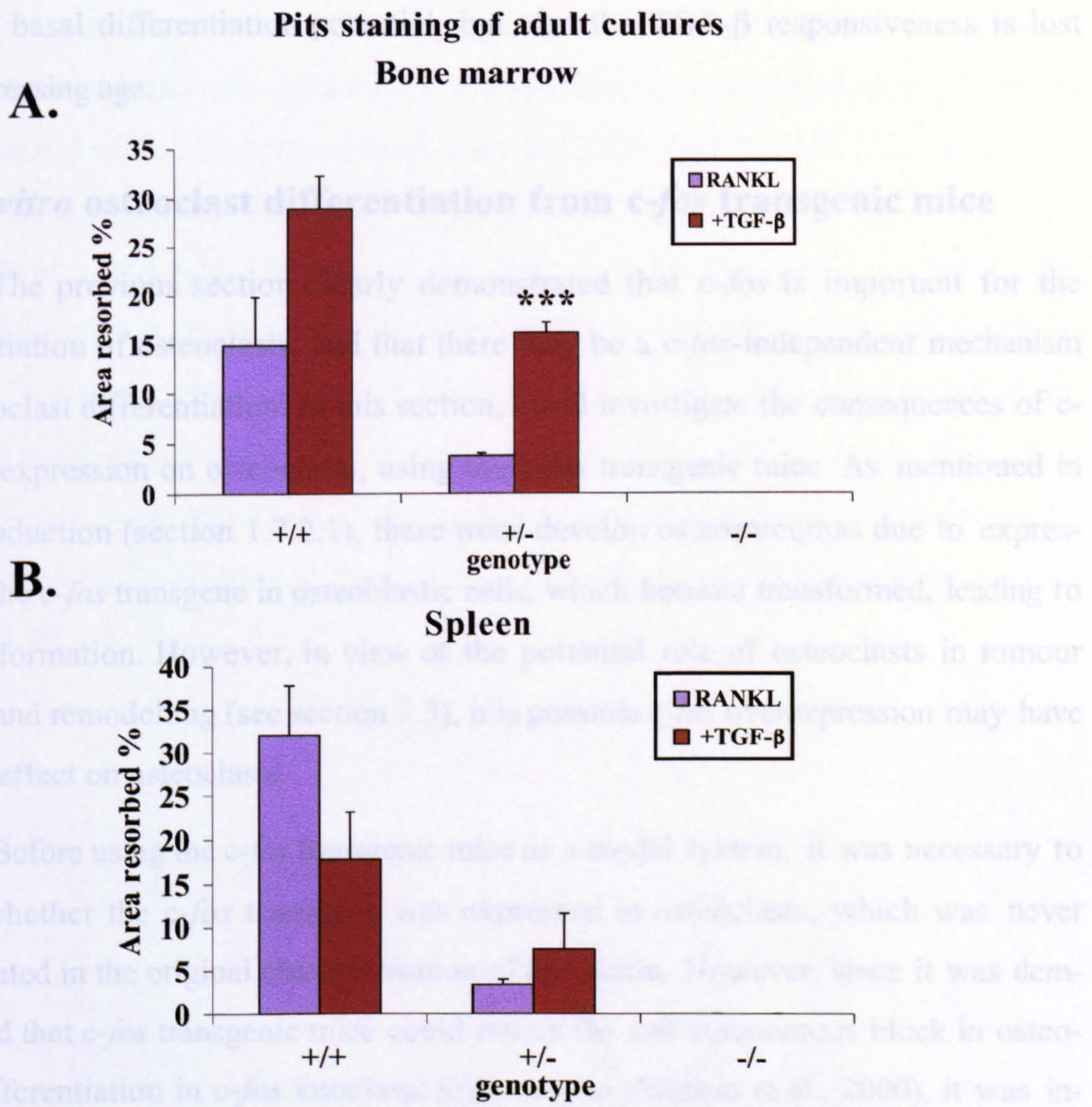



**Fig.4.3.13 TGF-β fails to induce resorptive activity in *c-fos* -/- adult bone marrow and spleen cultures.** M-CSF-dependent bone marrow (A) and spleen cells (B) from 2-month old male *c-fos* +/+, +/- and -/- mice were cultured with RANKL only, or together with TGF-β (1ng/ml) on dentine slices. Cells were fixed on day 10 and the percentage of resorbed area was quantified. Values are the mean ± SE of triplicate cultures from a representative experiment. *** P<0.001.

## 4.3.4 Summary

Taken together, the data presented in this section demonstrate that c-*fos* knockout cells can, under the appropriate conditions, differentiate into functional, osteoclast-like cells. This appears to be age-related, with newborn cells having a greater potential than adult cells. Moreover, the c-*fos*-independent differentiation potential appears to be restricted to bone marrow-derived cells, suggesting that there may be differences in the properties of M-CSF-dependent haematopoietic cells derived from bone marrow and spleen. Finally, TGF-β can partially rescue osteoclast differentiation in c-*fos* mutant cells, but only in young animals, suggesting that not

only the basal differentiation potential, but also the TGF-β responsiveness is lost with increasing age.

## 4.4 *In vitro* osteoclast differentiation from c-*fos* transgenic mice

The previous section clearly demonstrated that c-*fos* is important for the differentiation of osteoclasts, and that there may be a c-*fos*-independent mechanism for osteoclast differentiation. In this section, I will investigate the consequences of c-*fos* overexpression on osteoclasts, using the c-*fos* transgenic mice. As mentioned in the Introduction (section 1.7.2.1), these mice develop osteosarcomas due to expression of the c-*fos* transgene in osteoblastic cells, which become transformed, leading to tumour formation. However, in view of the potential role of osteoclasts in tumour growth and remodelling (see section 3.3), it is possible c-*fos* overexpression may have a direct effect on osteoclasts.

Before using the c-*fos* transgenic mice as a model system, it was necessary to check whether the c-*fos* transgene was expressed in osteoclasts, which was never investigated in the original characterisation of this strain. However, since it was demonstrated that c-*fos* transgenic mice could rescue the cell autonomous block in osteoclast differentiation in c-*fos* knockout mice *in vivo* (Matsuo et al., 2000), it was inferred that the c-*fos* transgene must be expressed in the osteoclast lineage. As shown in Fig.5.4, RT-PCR analysis of bone marrow-derived haematopoietic precursors confirmed that the c-*fos* transgene is expressed in the osteoclast lineage (see also chapter 5).

As described in the previous chapter, there were numerous osteoclasts present in tumour tissues from c-*fos* transgenic mice. In order to find out whether exogenous c-*fos* has direct effect on osteoclast differentiation, bone marrow cells from c-*fos* transgenic mice and their littermates were cultured *in vitro*.

### 4.4.1 Effects of exogenous c-*fos* on M-CSF and RANKL responsiveness during osteoclast differentiation

Since the c-Fos and AP-1 transcription factor lie downstream of both M-CSF and RANKL signalling (see Introduction, section 1.8.1, 1.8.3), initial experiments were to investigate whether c-*fos* transgenic osteoclast precursors showed a differen-

tial responsiveness to these osteoclastogenic cytokines. M-CSF-dependent non-adherent bone marrow cells from c-*fos* transgenic mice and their wild-type littermates were cultured with different concentrations of M-CSF and RANKL. The effects of different concentrations of RANKL in the presence of a fixed concentration of M-CSF showed a 2 to 3-fold increase in the number of TRAP-positive multinucleated cells generated from transgenic bone marrow cells compared to wild-type cells at RANKL concentrations greater than 5ng/ml (Fig.4.4.1 A). Similarly, M-CSF dose-response experiments in the presence of a fixed RANKL concentration showed an approximately 2-fold increase in TRAP-positive multinucleated cells from transgenic bone marrow cells compared to wild-type controls at all M-CSF concentrations (Fig.4.4.1.B).

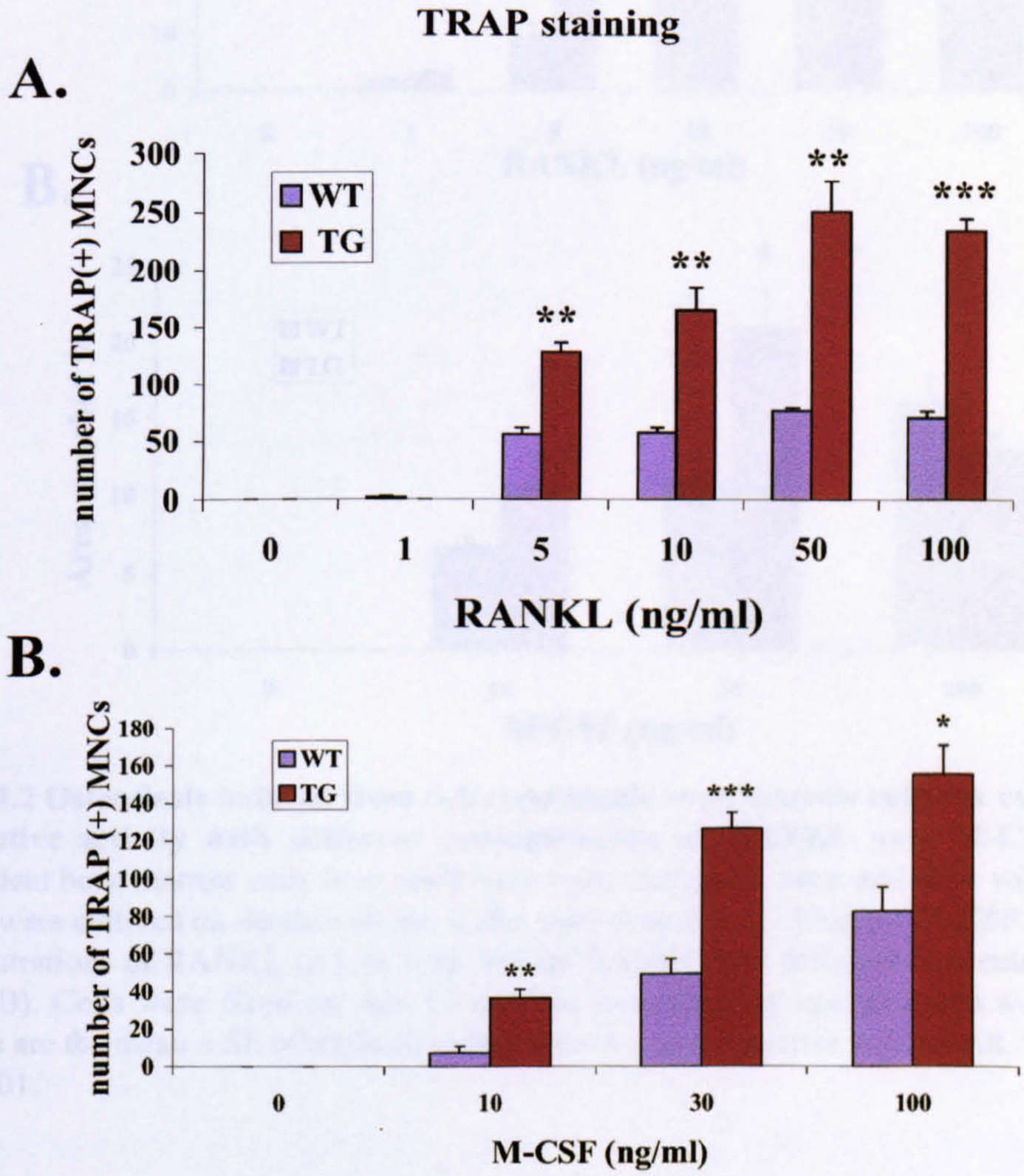



**Fig.4.4.1 Osteoclast precursors overexpressing *c-fos* exhibit increased responsiveness to M-CSF and RANKL.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type littermates were cultured in 96-well plates. Cells were treated with M-CSF (25ng/ml) and different concentrations of RANKL (A), or with RANKL (5ng/ml) and different concentrations of M-CSF (B) for 6 days. Cells were then fixed, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted. Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

The increase in the number of osteoclasts correlated well with an increase in resorption pit formation, with c-*fos* transgenic precursors showing a higher amount of resorption, with the exception of the highest doses of RANKL and M-CSF (Fig.4.4.2).

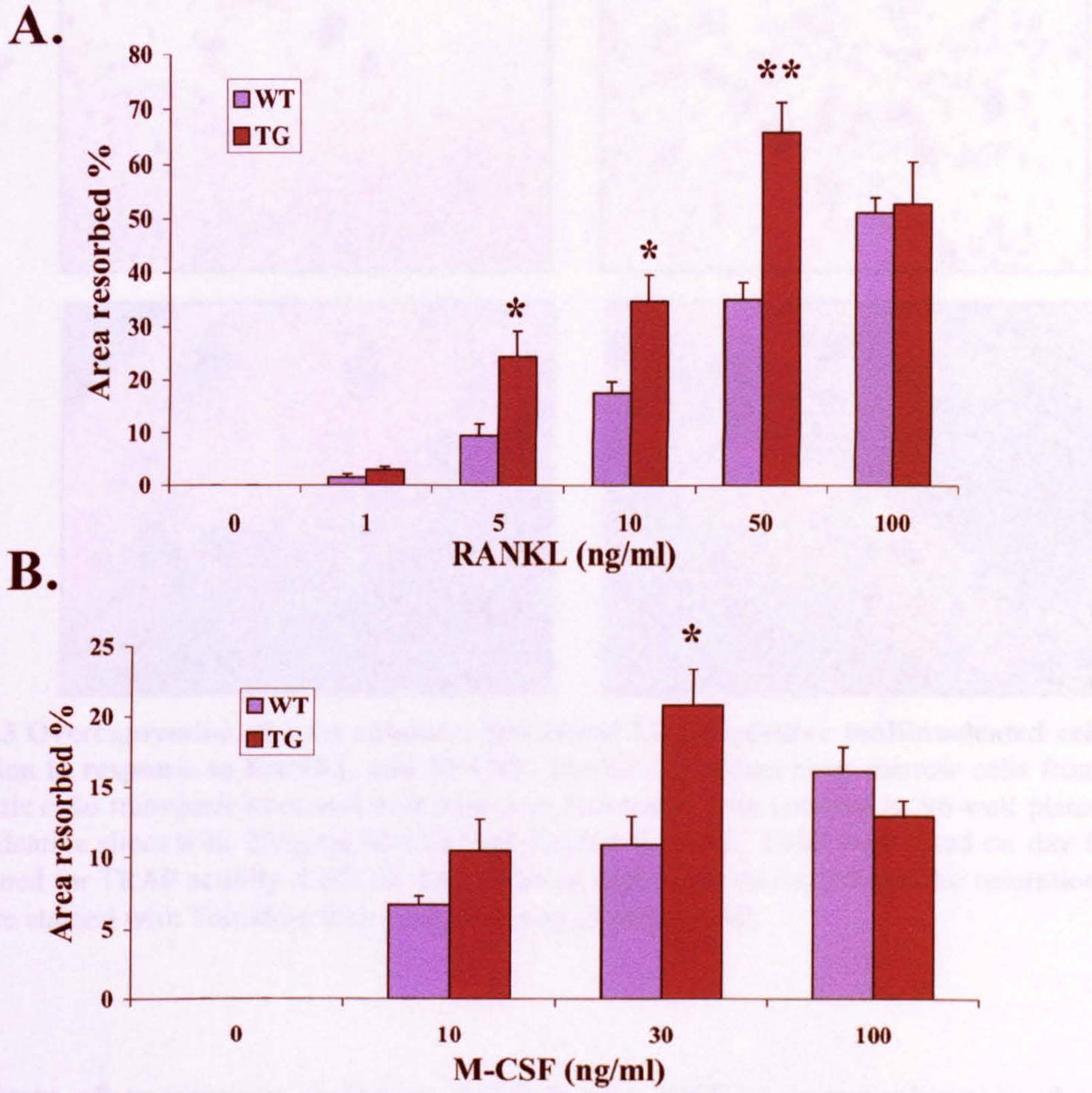



**Fig.4.4.2 Osteoclasts induced from *c-fos* transgenic bone marrow cultures exhibit higher resorptive activity with different concentrations of RANKL and M-CSF.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type litter-mates were cultured on dentine slices. Cells were treated with 25ng/ml M-CSF and different concentrations of RANKL (A), or with 5ng/ml RANKL and different concentrations of M-CSF (B). Cells were fixed on day 10 and the percentage of resorbed area was quantified. Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; ** $P<0.001$.

Thus, these data suggest that c-*fos* transgenic haematopoietic precursors show a greater sensitivity to the two essential osteoclast cytokines, M-CSF and RANKL. The effects of c-*fos* transgene expression in osteoclasts and resorption pit formation

under the standard culture conditions of 25ng/ml M-CSF and 5ng/ml RANKL are shown in Fig. 4.4.3.

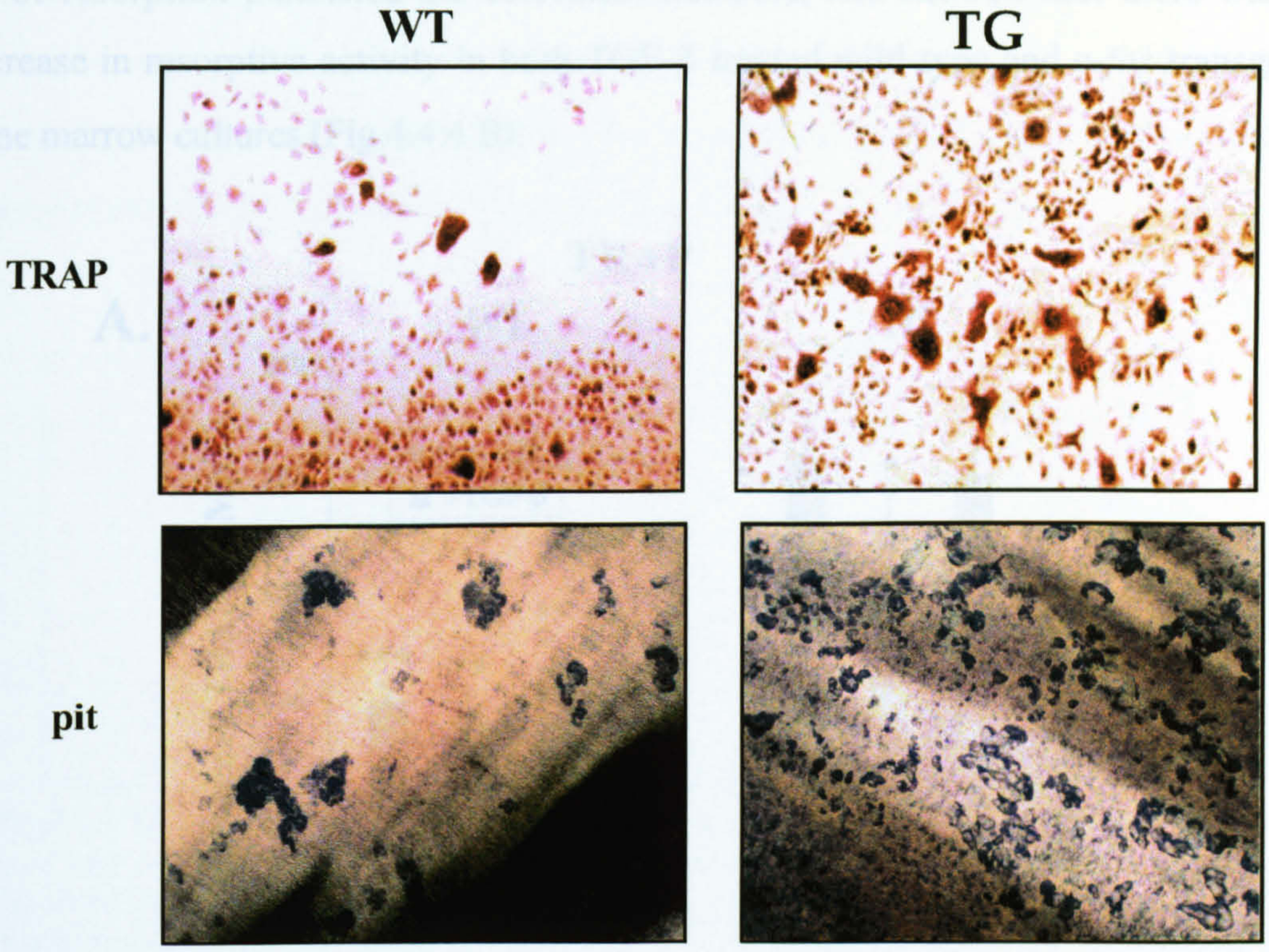



**Fig.4.4.3 Overexpression of *c-fos* enhances functional TRAP positive multinucleated cell formation in response to RANKL and M-CSF.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type littermates were cultured in 96-well plates and on dentine slices with 25ng/ml M-CSF and 5ng/ml RANKL. Cells were fixed on day 6 and stained for TRAP activity. Cells on dentine slices were fixed on day 10 and the resorption pits were stained with Toluidine Blue. Original magnification: ×10.

## 4.4.2 Effects of exogenous c-*fos* on TGF-β and TNF-α responsiveness during osteoclast differentiation

In view of the effects of TGF-β and TNF-α on osteoclast differentiation in c-*fos* knockout mice shown in the previous chapter, I next investigated whether over-expression of c-*fos* affects the responsiveness of osteoclast precursors to these cytokines. M-CSF-dependent non-adherent bone marrow cells from c-*fos* transgenic mice and their wild-type littermates were cultured with 1ng/ml of TGF-β in addition to M-CSF and RANKL for 5 to 8 days. At each time point, TGF-β increased the number of TRAP-positive multinucleated cells induced from both wild-type and c-*fos* transgenic bone marrow cultures (Fig.4.4.4 A). Although the overall numbers of os-

teoclasts were greater in c-*fos* transgenic cultures, the stimulatory effects of TGF-β appeared to be similar between wild-type and transgenic cells (Fig. 4.4.4 A). Analysis of resorption paralleled the osteoclast numbers, and showed that there was an increase in resorptive activity in both TGF-β treated wild-type and c-*fos* transgenic bone marrow cultures (Fig.4.4.4 B).

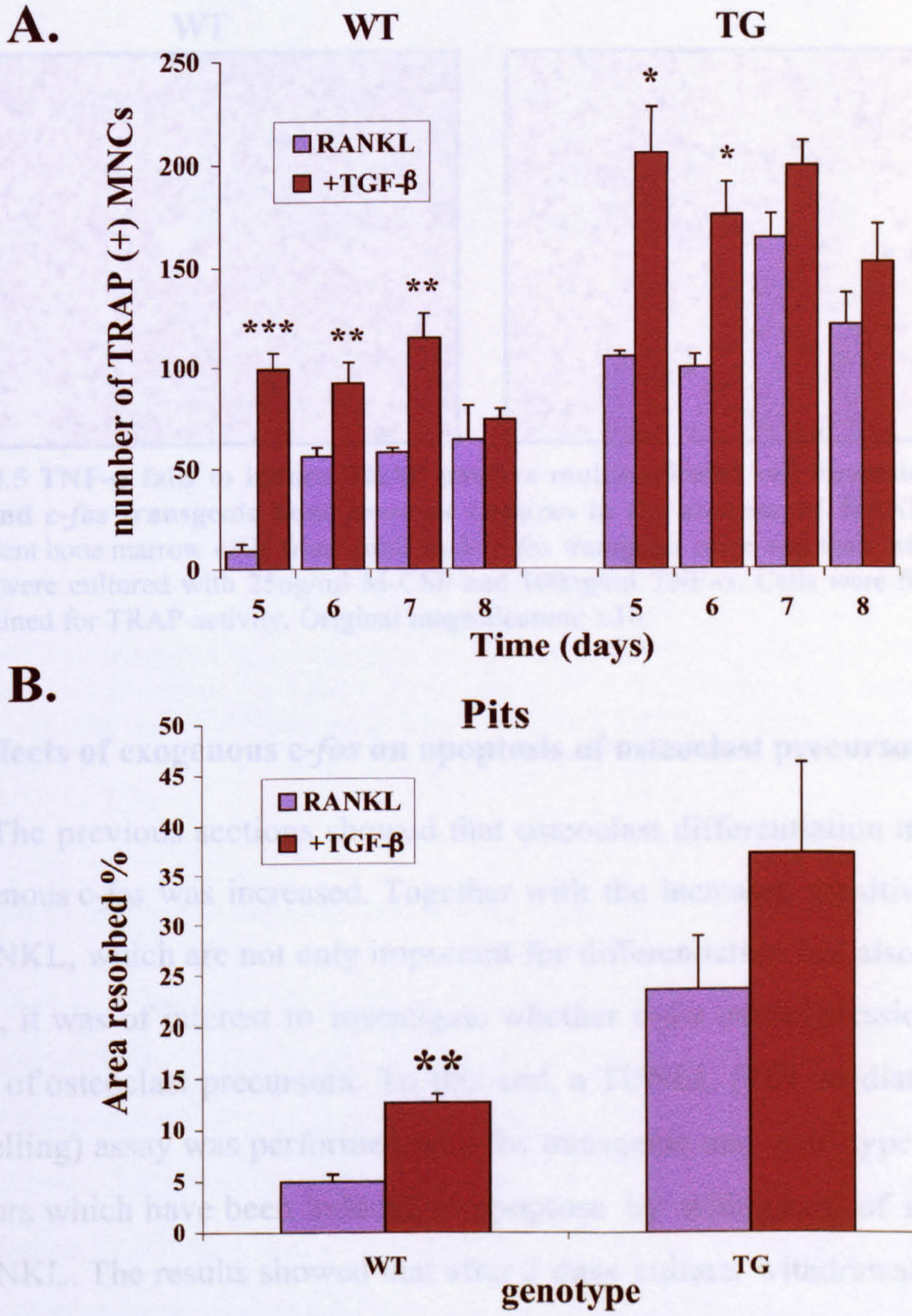



**Fig.4.4.4 TGF-β enhances TRAP positive multinucleated cell and resorption pit formation in both wild-type and *c-fos* transgenic bone marrow cultures.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type littermates were cultured with RANKL only, or together with TGF-β (1ng/ml) in 96-well plates or on dentine slices. Cells were fixed from day 5 to 8, stained for TRAP activity and the numbers of TRAP positive multinucleated cells were counted (A). Cells on dentine slices were fixed on day 9 and the percentage of resorbed area was quantified. Values are the mean ± SE of triplicate cultures from a representative experiment. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

M-CSF-dependent non-adherent bone marrow cells from c-*fos* transgenic mice and their wild-type littermates were also cultured with TNF-α and 25ng/ml of M-CSF in the absence of RANKL to find out whether overexpression of c-*fos* affects the responsiveness of osteoclast precursors to TNF-α. Interestingly, no TRAP positive multinucleated cells were formed in the absence of RANKL from both wild-type and c-*fos* transgenic bone marrow culture (Fig.4.4.5).

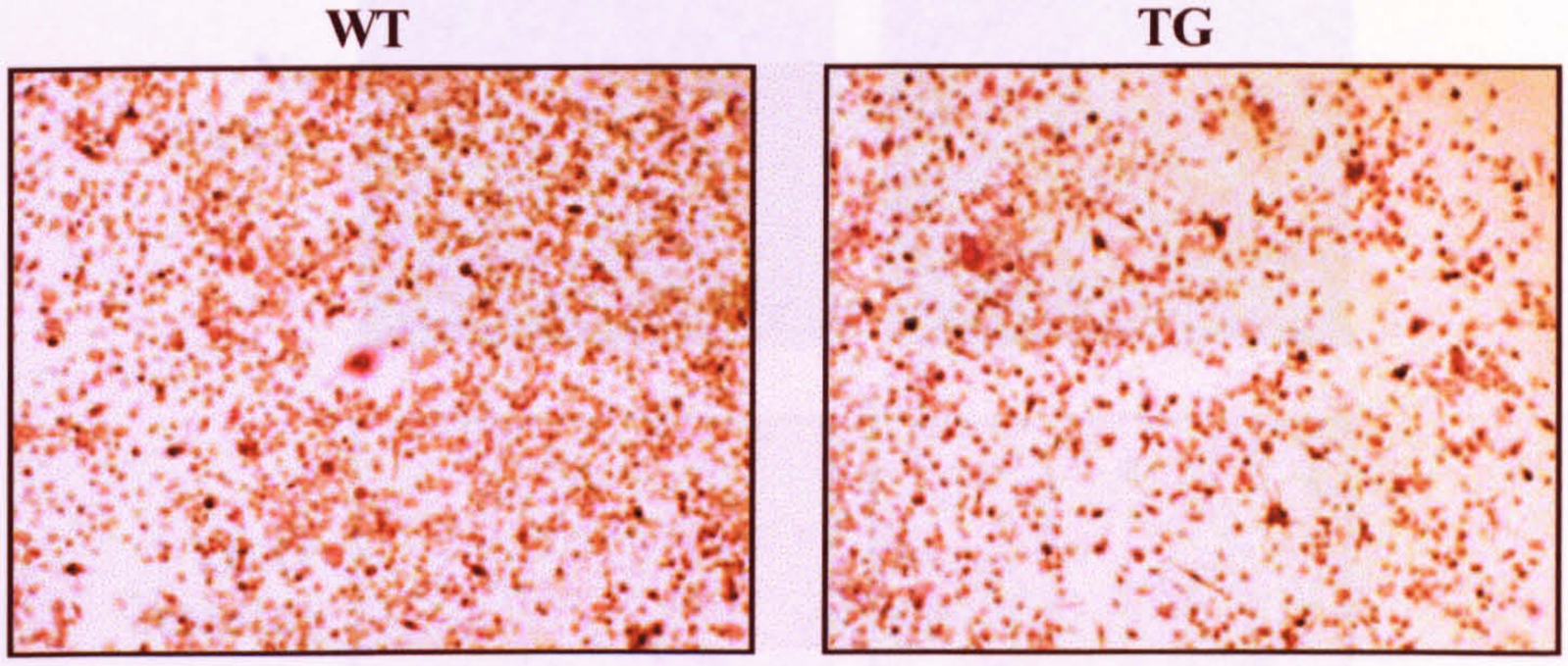



**Fig.4.4.5 TNF-α fails to induce TRAP positive multinucleated cell formation from wild type and *c-fos* transgenic bone marrow cultures in the absence of RANKL.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type littermates were cultured with 25ng/ml M-CSF and 100ng/ml TNF-α. Cells were fixed on day 6 and stained for TRAP activity. Original magnification: ×10.

### 4.4.3 Effects of exogenous c-*fos* on apoptosis of osteoclast precursors

The previous sections showed that osteoclast differentiation in the presence of exogenous c-*fos* was increased. Together with the increased sensitivity to M-CSF and RANKL, which are not only important for differentiation but also for osteoclast survival, it was of interest to investigate whether c-*fos* overexpression affected the survival of osteoclast precursors. To this end, a TUNEL (TdT-mediated dUTP nick end labelling) assay was performed on c-*fos* transgenic and wild-type bone marrow precursors which have been induced to apoptose by withdrawal of serum, M-CSF and RANKL. The results showed that after 2 days culture, withdrawal of factors for a further 24 hours caused a significant increase in the number of TUNEL-positive cells, but there were no obvious differences between wild-type and transgenic cultures (Fig.4.4.5). These results suggest that increased survival of osteoclast lineage cells is unlikely to be the main reason for the enhanced osteoclast differentiation seen in c-*fos*-overexpressing cells.

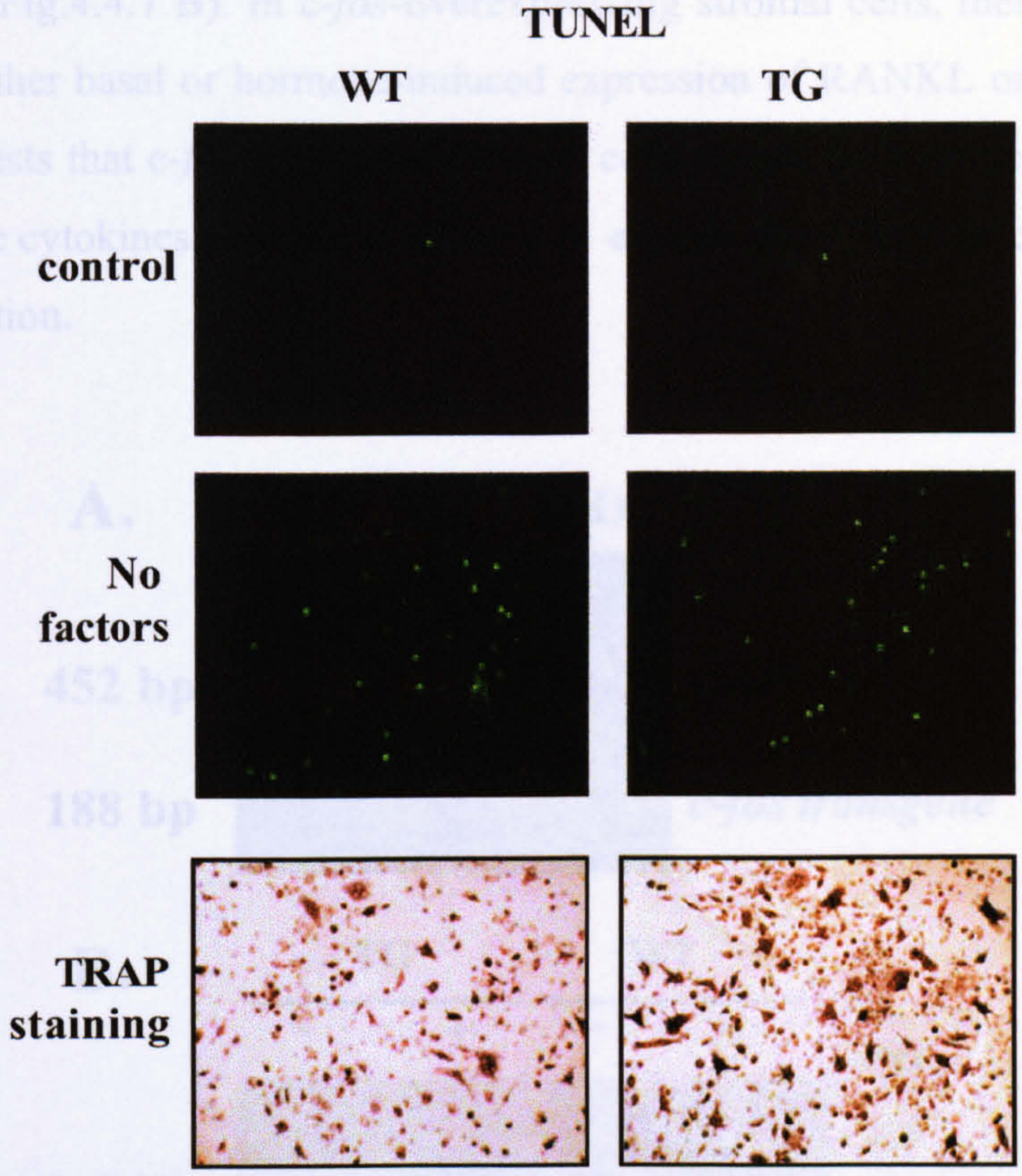



**Fig.4.4.6 No differences in the amount of apoptotic cells between *c-fos* transgenic and wild-type precursors.** M-CSF-dependent bone marrow cells from adult male *c-fos* transgenic mice and their wild-type littermates were cultured with M-CSF and RANKL (30ng/ml) in 24-well plates on coverslips for 2 days. Serum and growth factors were then withdrawn for 24 hours, and cells were fixed and stained for TRAP and TUNEL activity. Original magnification: ×10.

### 4.4.4 Effects of exogenous c-*fos* on RANKL and OPG expression

Finally, since in primary bone marrow cultures, there may be some contaminating stromal cells remaining in the cultures, it was important to confirm that the effect of exogenous c-*fos* on osteoclast differentiation is not secondary to the effect on osteoblasts. This was done by isolating stromal cells from c-*fos* transgenic mice and wild-type littermates, and examining the expression of RANKL and OPG. First, analysis of transgene expression carried out by RT-PCR, showed that exogenous *c-fos* was expressed in transgenic stromal cells, but was absent in the wild-type stromal cells (Fig.4.4.7 A). The transgene levels were quite low and could not be detected readily by Northern blotting. Analysis of RANKL and OPG expression by Northern blot analysis showed that in wild-type mice, addition of $1,25(OH)_2D_3$ and Dexamethasone stimulated the expression of RANKL but inhibited OPG expression, as

expected (Fig.4.4.7 B). In c-*fos*-overexpressing stromal cells, there were no differences in either basal or hormone-induced expression of RANKL or OPG (Fig.4.4.6). This suggests that c-*fos* transgenic stromal cells do not have altered levels of osteoclastogenic cytokines and this is unlikely to explain the effects of c-*fos* on osteoclast differentiation.

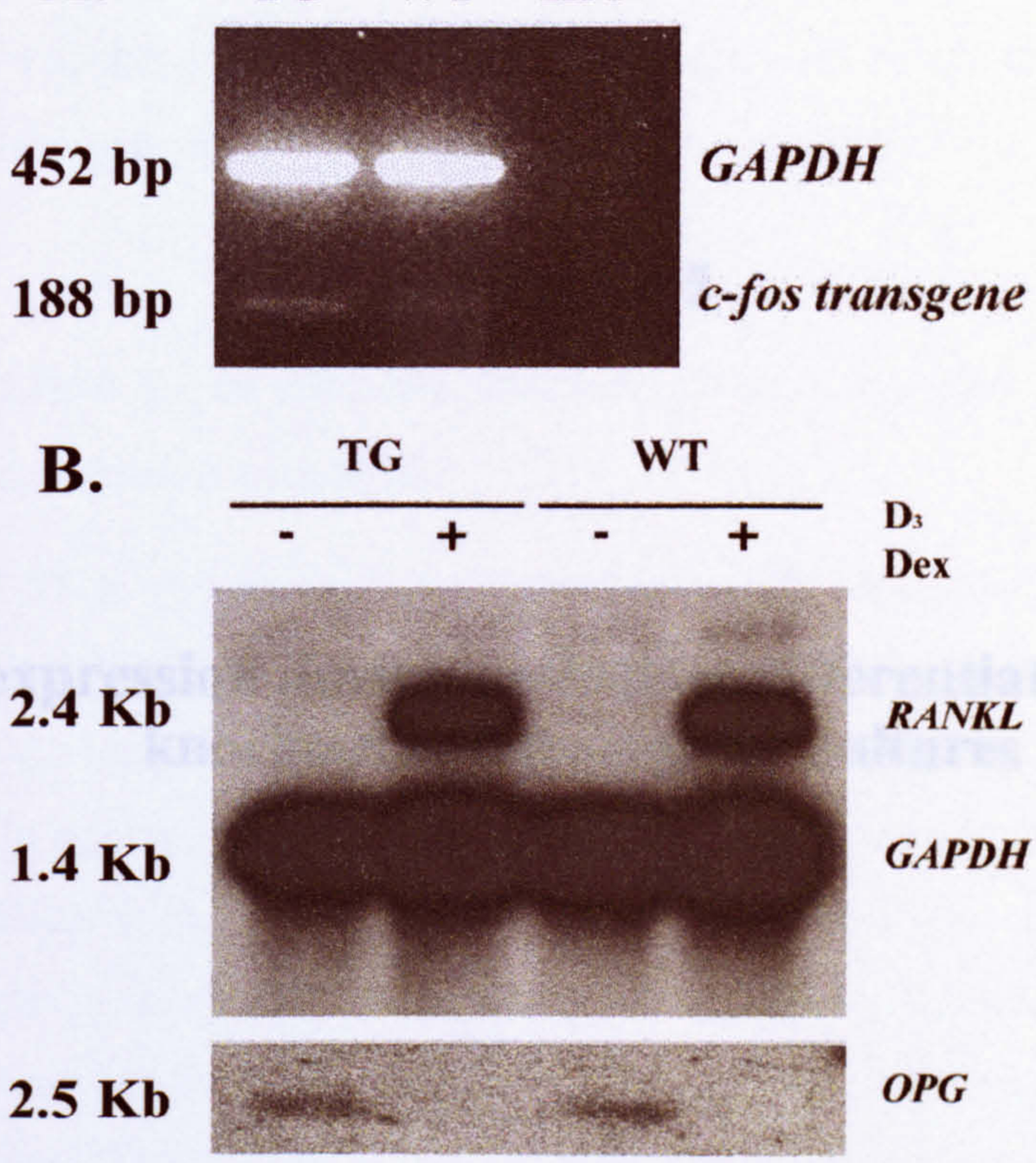



**Fig.4.4.7 Expression of RANKL and OPG in c-Fos transgenic stromal cells.** Stromal cells were isolated from *c-fos* transgenic mice and their wild-type littermates. Cells were treated with or without $10^{-8}$M 1,25 $(OH)_2D_3$ and $10^{-8}$M Dexamethasone for three days. The expression of *c-fos* transgene was investigated by RT-PCR (A), and the expression of *RANKL* and *OPG* was investigated by Northern blot (B). *GAPDH* was used as a loading control.

## 4.5 Discussion

The results of this chapter must be viewed in the context of the molecular data provided in the next chapter. Therefore, the results of both chapters will be discussed in one section at the end of chapter 5 (see section 5.7).

# Chapter 5

# Gene expression during osteoclast differentiation in *c-fos* knockout and transgenic cultures

## 5.1 Introduction

The previous chapter used tissue culture experiments to show that c-*fos* knockout precursors can, to a limited extent, differentiate into functional osteoclasts, and that overexpression of c-*fos* can increase the sensitivity of osteoclasts precursors to M-CSF and RANKL. In order to investigate the possible mechanism by which *c-fos* affects osteoclast differentiation, the expression of osteoclast marker genes and signalling factors for osteoclast differentiation was analysed by semi-quantitative RT-PCR in *c-fos* knockout and transgenic cell cultures, both before differentiation in M-CSF-dependent precursors, and after induction of differentiation by M-CSF and RANKL.

## 5.2 Expression of osteoclast marker genes in *c-fos* knockout bone marrow and spleen cultures

Total RNA was isolated before and after differentiation from newborn, 2-3 week-old and adult *c-fos* +/+ and -/- male bone marrow and spleen cultures. RT-PCR of the osteoclast marker genes TRAP and CTR showed that TRAP was expressed at lower levels in c-*fos* -/- cultures, especially in newborn cultures which can form functional osteoclasts (Fig.5.1 A-C), although the expression was quite variable. Since TRAP expression *in vitro* can be an inconsistent marker, especially at the RT-PCR level, I also analysed the expression of CTR, which is the best marker of osteoclasts. First, CTR was expressed in all wild-type samples only after differentiation, validating the use of this as a good marker. CTR expression was clearly observed in c-*fos* mutant newborn cultures, and this was at a lower level than in wild-type controls (Fig.5.1 A), which correlated well with their differentiation capacity shown in the previous chapter. In post-natal day 18 cultures, CTR was not easily detectable in mutant bone marrow cultures after differentiation, which is expected since only a low number of mononuclear TRAP-positive cells were observed in these cultures (Fig.5.1 B; see also Fig.4.2.3, 4.2.4). In 2 month-old cultures, no CTR expression was detected either in bone marrow or spleen-derived precursors (Fig.5.1 C), consistent with their lack of ability to form functional osteoclasts. Thus, in newborn *c-fos* -/- cultures which formed functional osteoclasts, this was confirmed at the molecular level with CTR expression.

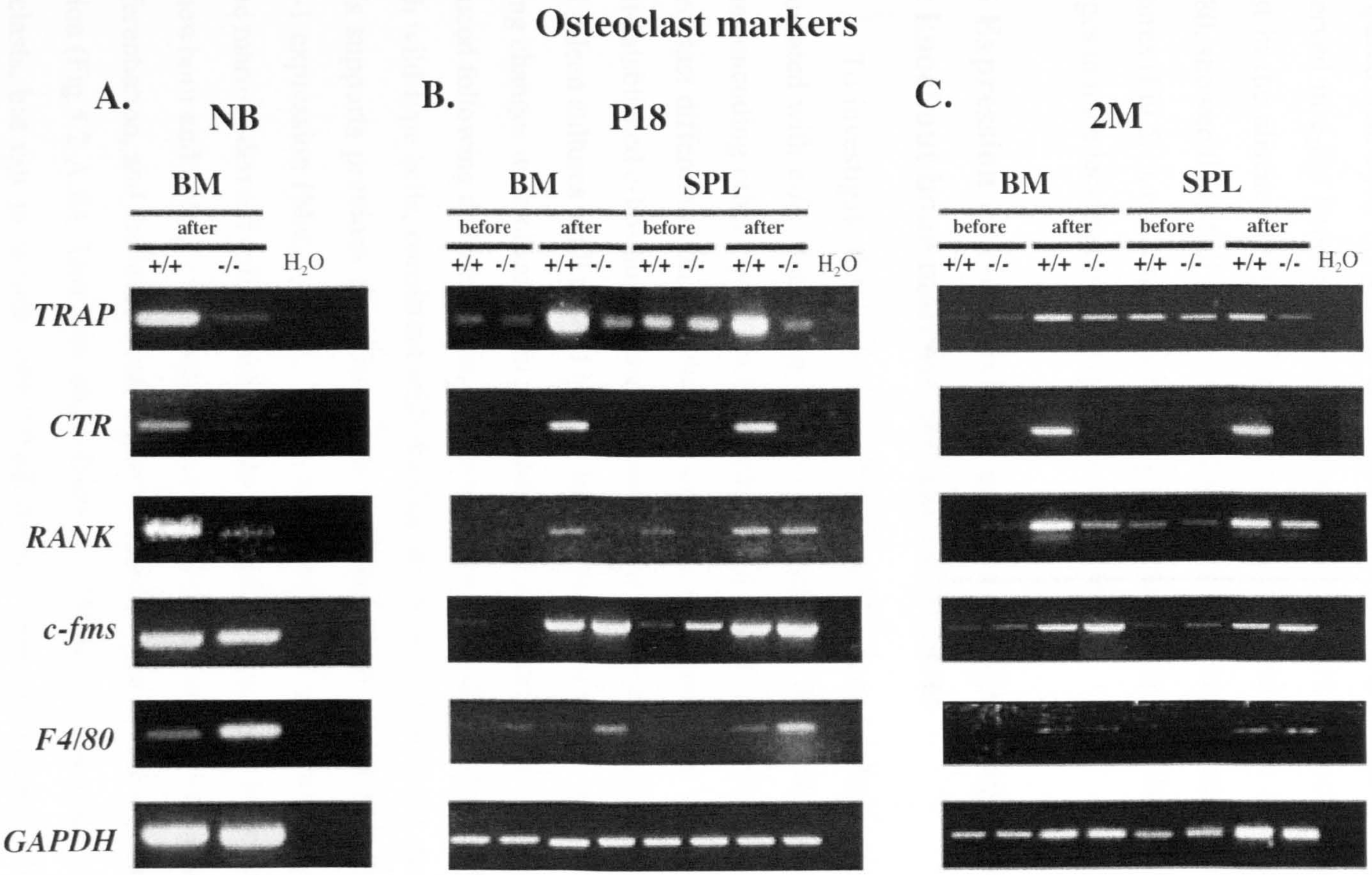



**Fig.5.1 Expression of osteoclast and macrophage marker genes in differentiation cultures of *c-fos* knockout haematopoietic cells.** M-CSF-dependent bone marrow and spleen cells isolated from *c-fos* +/+, +/- newborn (A), postnatal day 18 (P18) (B) and 2-month old adult male mice (C) were cultured under standard osteoclast differentiation conditions. Total RNA was isolated from M-CSF-dependent non-adherent precursors prior to the addition of RANKL (before differentiation) and from cells cultured for 5-6 days in the presence of RANKL (after differentiation). RT-PCR analysis of *TRAP*, *CTR*, *RANK*, *c-fms* and *F4/80* expression was performed. *GAPDH* was used as a loading control.

Analysis of the receptors for RANKL and M-CSF, RANK and c-*fms*, respectively, showed that both genes were upregulated after differentiation of wild-type cells (Fig.5.1). However, in c-*fos* -/- cultures, whereas no significant changes in c-*fms* expression were observed, *RANK* expression was reduced in *c-fos* mutant cells when compared with *c-fos* +/+ cells (Fig.5.1 A-C). The fact that *RANK* expression was still observed in c-*fos* knockout cells suggests that putative osteoclast precursors still exist in the absence of c-*fos*. Finally, analysis of a definitive macrophage marker, F4/80, showed that at all ages tested, F4/80 expression was increased in c-*fos* mutant cultures (Fig.5.1), confirming previously published results that there are more macrophages in mice lacking c-*fos* (Grigoriadis et al., 1994).

## 5.3 Expression of c-Fos-related genes and signalling molecules in *c-fos* knockout bone marrow and spleen cultures

To investigate the expression of genes which may lie downstream of, or are associated with c-*fos* during osteoclast differentiation, RT-PCR was performed for genes encoding other c-*fos* family members and signalling molecules important for osteoclast differentiation. In wild-type cultures, the expression of *fra*-1, which is a well-established c-Fos target gene, increased after differentiation in both bone marrow and spleen cultures at all ages (Fig.5.2). In the absence of c-Fos, however, some interesting changes were observed: in adult-derived c-*fos* -/- cells, *fra*-1 expression was not induced following differentiation, and levels were lower after differentiation compared with wild-type cells, consistent with the fact that it is a c-Fos target gene (Fig.5.2 C). This supports previous data that adult *c-fos* knockout spleen cells have decreased *fra*-1 expression (Matsuo et al., 2000), and this is now also shown here with adult bone marrow-derived cells, which also do not form osteoclasts. In contrast, however, in newborn and young *c-fos* knockout cells, *fra*-1 expression was stimulated during differentiation, and the levels were higher than wild-type controls following differentiation (Fig.5.2 A,B). This was only observed in bone marrow cells, which form osteoclasts, but not in spleen cells, which do not form osteoclasts. The other c-*fos*-related gene, *fra*-2, did not show any significant changes in either bone marrow or spleen populations at all ages examined (Fig.5.2).

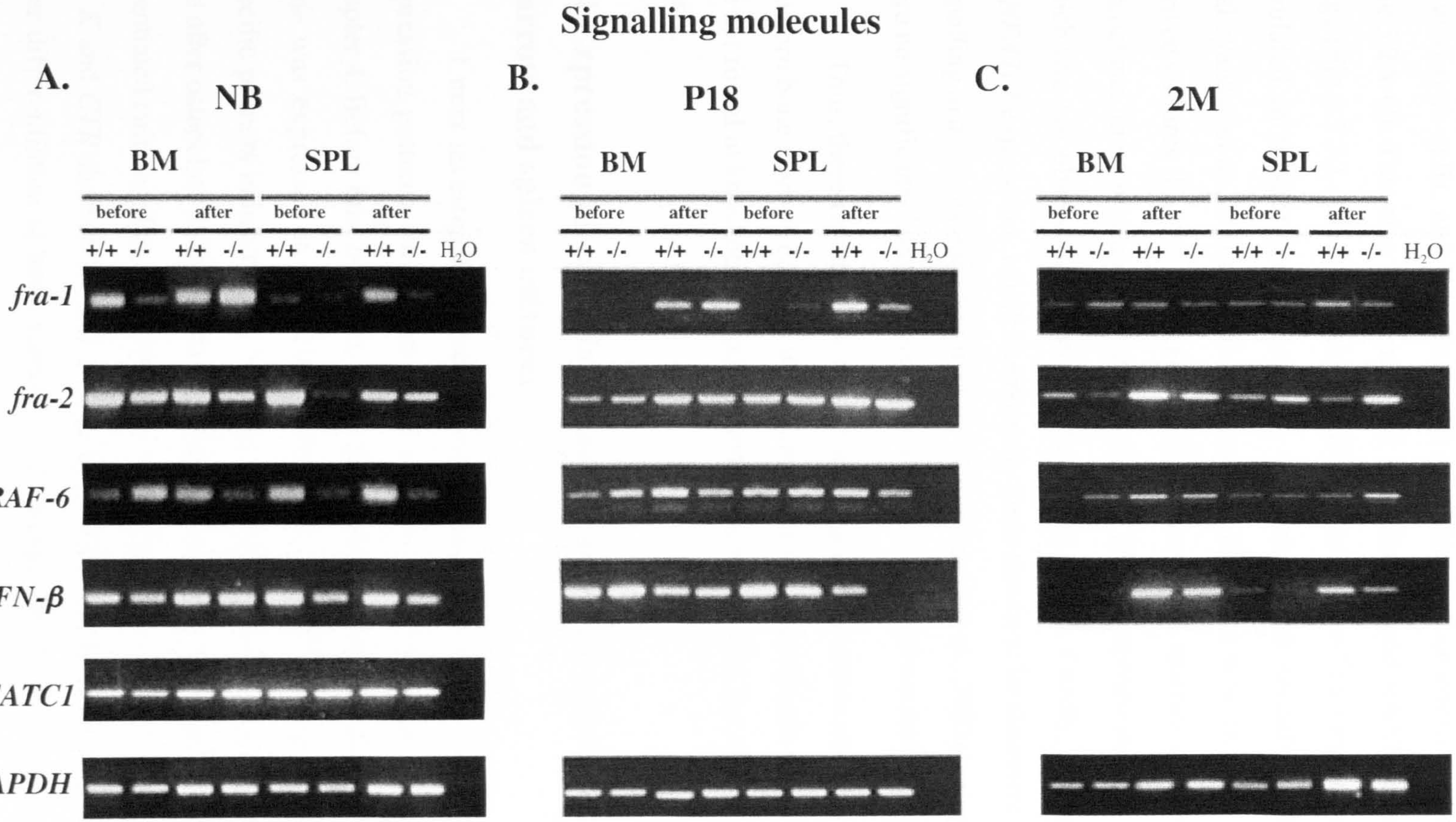



**Fig.5.2 Expression of osteoclast signalling molecules and c-Fos-related genes in differentiation cultures of *c-fos* knockout haematopoietic cells.** M-CSF-dependent bone marrow and spleen cells isolated from *c-fos* +/+ and -/- newborn (A), postnatal day 18 (P18) (B) and 2-month old adult male mice (C) were cultured under standard osteoclast differentiation conditions. Total RNA was isolated from M-CSF-dependent non-adherent precursors prior to the addition of RANKL (before differentiation) and from cells cultured for 5-6 days in the present of RANKL (after differentiation). RT-PCR analysis of *fra-1*, *fra-2*, *TRAF-6*, *IFN*-b and *NFATc1* expression was performed. *GAPDH* was used as a loading control.

The expression of TRAF-6, which is downstream of RANK receptor signalling, was generally reduced after differentiation of c-*fos* knockout cultures compared to wild-type cells, and this was most prominent in newborn and young cultures (Fig.5.2A-C). This also correlates well with the reduced RANK expression at this stage (Fig.5.1 A). The expression of *IFN-β*, another c-Fos target gene, was down-regulated in mutant spleen cultures, consistent with recently published results (Takayanagi et al., 2002b), and this was also observed now in adult bone marrow-derived cultures (Fig.5.2 C). In newborn knockout bone marrow cultures, which form osteoclasts, there was a slight upregulation of *IFN-β* expression after differentiation, which was not observed in spleen cultures (Fig.5.2 A). Finally, preliminary analysis of *NFATc1* expression, which has recently been shown to be downstream of RANKL signalling in a c-Fos-dependent manner (Takayanagi et al., 2002a), showed that there were no significant changes in newborn mutant bone marrow and spleen cultures.

Thus, these results show some unique gene expression patterns specifically in newborn bone marrow cultures which have retained osteoclastic potential, and which are not found in knockout cell populations which have lost the ability to form osteoclasts.

## 5.4 Expression of osteoclast marker genes in *c-fos* transgenic bone marrow and spleen cultures

I next investigated whether overexpression of c-*fos* resulted in altered gene expression patterns which correlated with the differentiation effects observed in chapter 4. Before this, however, it was important to demonstrate that the c-*fos* transgene was expressed in osteoclast precursors. RT-PCR analysis using transgene-specific primers indicated that the c-*fos* transgene was indeed expressed both before and after osteoclast differentiation, although the levels were apparently lower in differentiated cultures (Fig. 5.4). Analysis of the osteoclast marker genes *TRAP*, *cathepsin K* and *CTR* showed that all these genes were expressed at slightly higher levels after differentiation of bone marrow cells derived from c-*fos* transgenic mice compared to wild-type littermate controls (Fig. 5.3). This is consistent with the increased number of osteoclasts seen in the differentiation experiments shown in chapter 4. Interestingly, the expression of the receptor *RANK* showed higher levels in transgenic

cultures both before and after differentiation (Fig.5.3). This may explain the increased sensitivity of transgenic osteoclast precursors to RANKL treatment which was shown in chapter 4 (Fig.4.4.1 A), and raises the possibility that RANK expression may be regulated, either directly or indirectly, by c-Fos. In contrast, no changes in c-*fms* expression were observed between transgenic and wild-type cultures (Fig. 5.3), suggesting that the increases responsiveness to M-CSF observed earlier (Fig. 4.4.1 B) may lie downstream of c-*fms* receptor signalling.

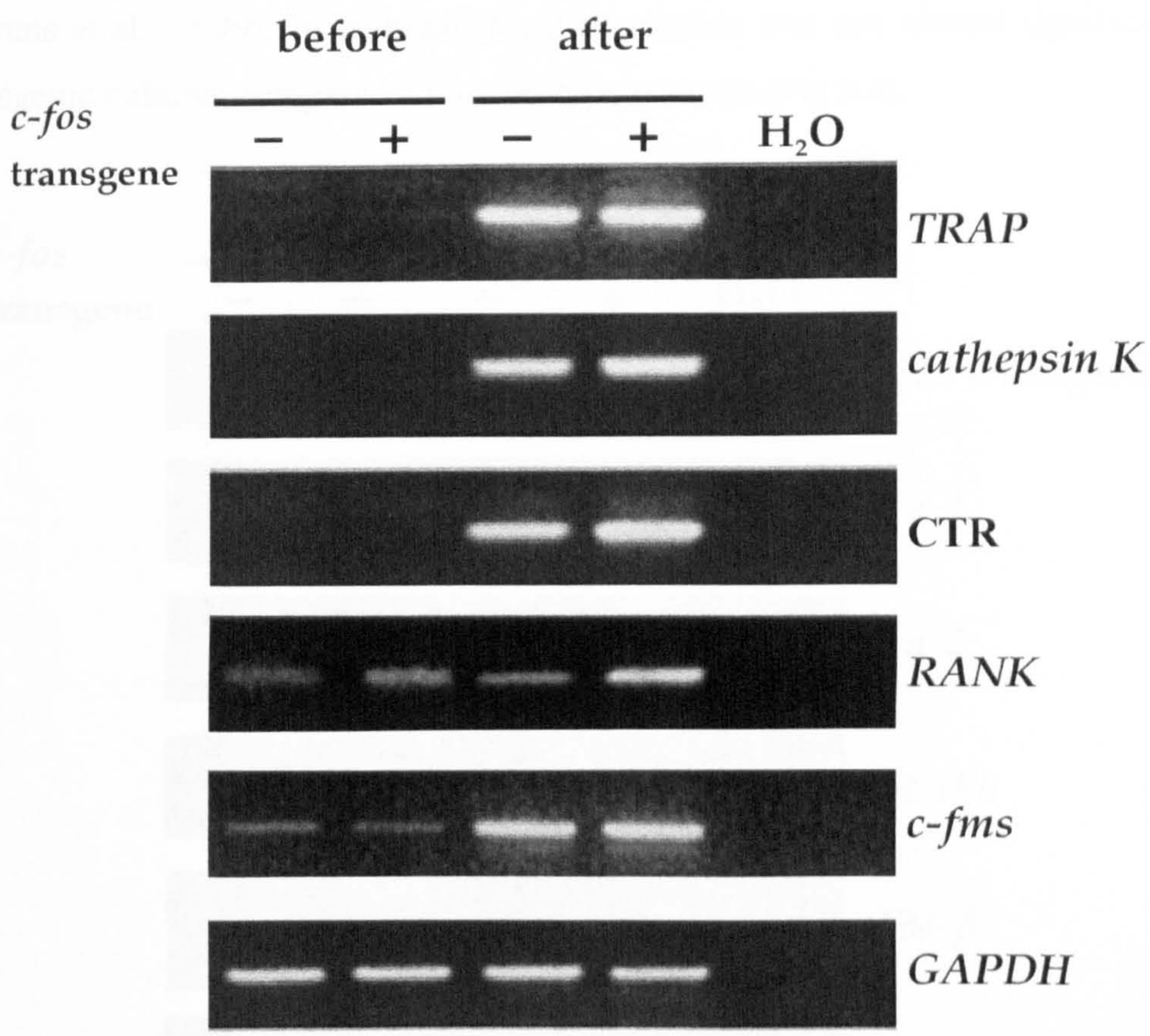



**Fig.5.3 Expression of osteoclast and macrophage marker genes in differentiation cultures of c-Fos transgenic haematopoietic cells.** M-CSF-dependent bone marrow cells isolated from adult male c-Fos-transgenic mice and their wild-type littermates were cultured under standard osteoclast differentiation conditions. Total RNA was isolated from M-CSF-dependent non-adherent precursors prior to the addition of RANKL (before differentiation) and from cells cultured for 5-6 days in the presence of RANKL (after differentiation). RT-PCR analysis of *TRAP*, *cathepsin K*, *CTR*, *RANK* and *c-fms* expression was performed. *GAPDH* was used as a loading control.

## 5.5 Expression of c-Fos-related genes and signalling molecules in *c-fos* transgenic bone marrow and spleen cultures

Analysis of the c-Fos target gene, *fra*-1, demonstrated that the expression of *fra*-1 was upregulated in transgenic M-CSF-dependent precursors before differentiation, and this increase was maintained after osteoclast differentiation (Fig.5.4). This implicates *fra*-1 as a downstream gene which may explain why c-*fos*-overexpression causes enhanced osteoclast differentiation, and is consistent with the previously published data that overexpression of *fra*-1 can stimulate osteoclast differentiation (Owens et al., 1999). In contrast, *fra*-2 expression was not altered significantly in transgenic cultures compared with wild-type controls (Fig.5.4).

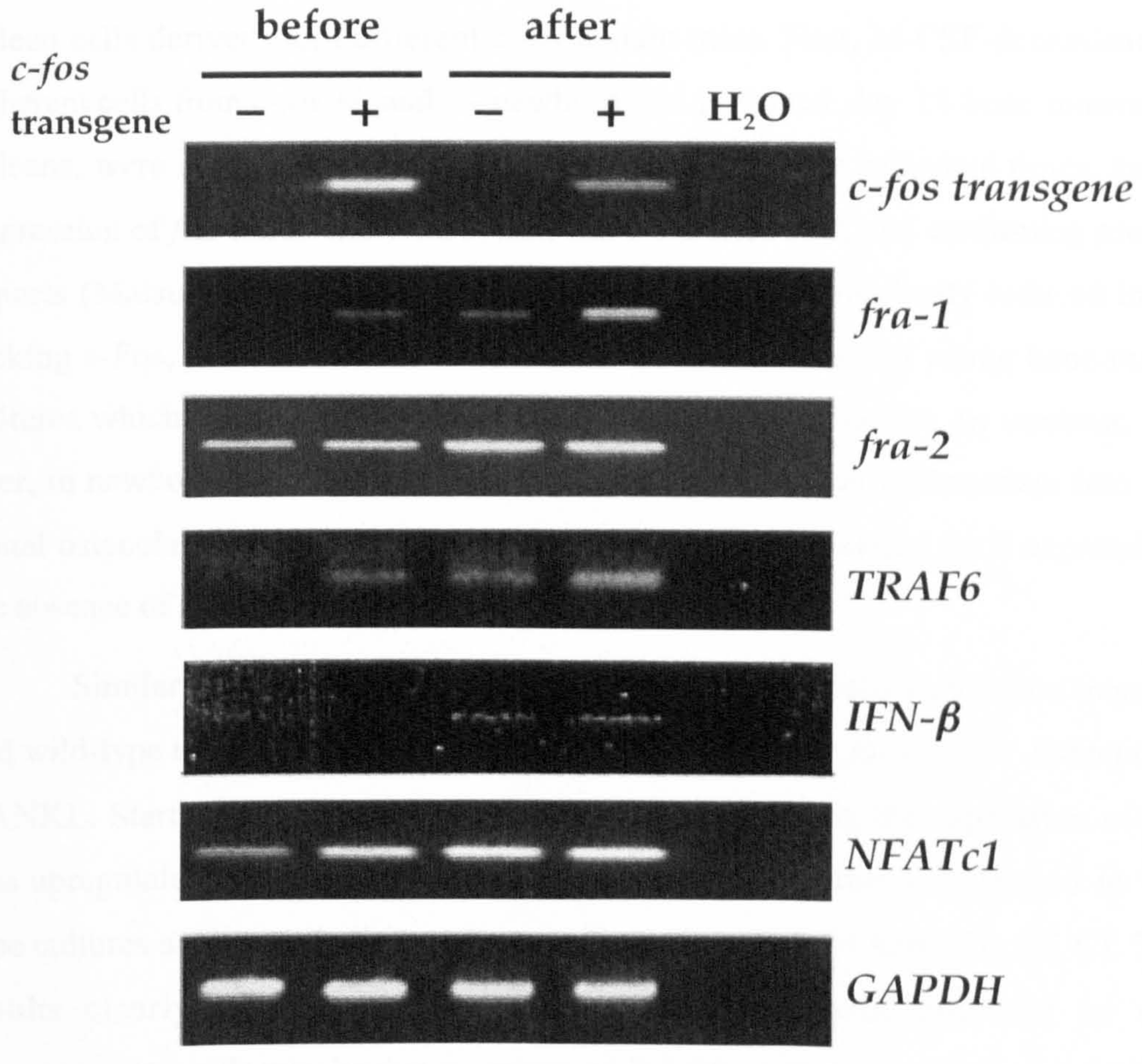



**Fig.5.4 Expression of osteoclast signalling molecules and c-Fos-related genes in differentiation cultures of c-Fos transgenic haematopoietic cells.** M-CSF-dependent bone marrow cells isolated from adult male c-Fos-transgenic mice and their wild-type littermates were cultured under standard osteoclast differentiation conditions. Total RNA was isolated from M-CSF-dependent non-adherent precursors prior to the addition of RANKL (before differentiation) and from cells cultured for 5-6 days in the present of RANKL (after differentiation). RT-PCR analysis of *c-fos*-transgene, *fra-1*, *fra-2*, *TRAF-6*, *IFN-β* and *NFATc1* expression was performed. *GAPDH* was used as a loading control.

Analysis of *TRAF-6* expression also showed significant increases in expression in c-*fos*-overexpressing cultures both before and after differentiation (Fig.5.4), and this was consistent with the increased expression of the receptor *RANK* observed earlier (Fig.5.3). On the other hand, no significant changes were observed in the expression of *IFN-β* and *NFATc1* (Fig.5.4).

## 5.6 Time course of *fra-1* expression in osteoclast precursors after RANKL stimulation

Finally, to further analyse the role of *fra*-1 in the context of varying c-*fos* levels, and to compare with previous published data, I analysed the induction of *fra*-1 expression following RANKL treatment of M-CSF-dependent bone marrow and spleen cells derived from different c-*fos* mutant mice. First, M-CSF-dependent non-adherent cells from *c-fos* +/- and -/- newborn and postnatal day 18 bone marrow and spleens, were stimulated with M-CSF and RANKL for the indicated times, and the expression of *fra-1* was analysed by RT-PCR. As expected, and confirming previous reports (Matsuo et al., 2000), the induction of *fra*-1 was markedly reduced in cells lacking c-Fos, and this was observed in the spleen cultures and young bone marrow cultures which do not make multinucleated osteoclasts (Fig.5.5). In contrast, however, in newborn knockout bone marrow cultures which can differentiate into functional osteoclasts, there was no reduction in RANKL-stimulated *fra*-1 expression in the absence of c-*fos* (Fig.5.5 A).

Similar experiments were done with bone marrow cells from *c-fos* transgenic and wild-type mice to investigate the effects of exogenous *c-fos* on *fra-1* induction by RANKL. Starting as early as 30min after RANKL addition, the expression of *fra-1* was upregulated in *c-fos*-overexpressing bone marrow cultures compared to wild-type cultures at all time points, and reached a peak at about 1 hour (Fig.5.5 C). These results clearly suggest that the increase in osteoclastic potential in c-*fos*-overexpressing cells may be due to enhanced RANK receptor expression, which lead to increased responsiveness to RANKL and activation of downstream targets such as *fra*-1.

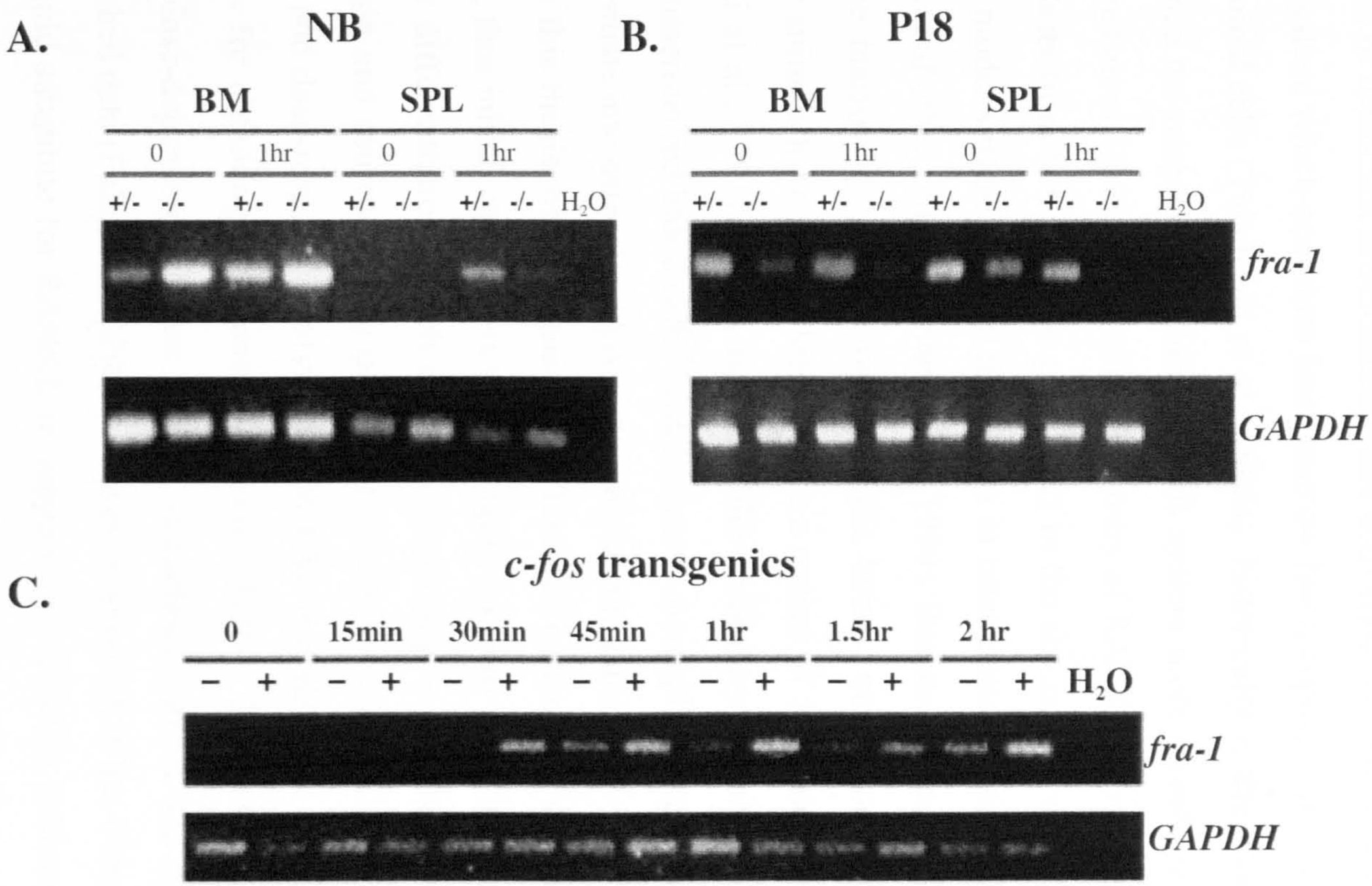



**Fig.5.5 Time course of *fra-1* induction by RANKL in cultures of osteoclast precursor cells from c-Fos knockout and transgenic mice.** M-CSF-dependent bone marrow and spleen cells from *c-fos* +/- and -/- newborn (A), and postnatal day 18 (P18) mice (B), and bone marrow cells from male c-Fos transgenic mice and their wild-type littermates (C) were cultured with 25ng/ml M-CSF overnight. Total RNA was isolated from non-adherent cells (time 0) and from cells stimulated with 5ng/ml RANKL for the indicated times. RT-PCR analysis of *fra-1* expression was performed. *GAPDH* was used as a loading control.

## 5.7 Discussion

### 5.7.1 Establishment of an *in vitro* culture system

Many *in vitro* model systems have been established so far to study the mechanisms of osteoclast differentiation and activation. Among all the methods, the coculture system, which combines bone marrow haematopoietic precursors and supportive stromal cells (Takahashi et al., 1988a), historically is the most widely employed, since the osteoclasts induced in this system more closely resemble the *in vivo* circumstances. However, after the discovery of RANKL, it is possible to generate osteoclasts from bone marrow precursors in the absence of stromal cells, which provides a model system to study the events in osteoclastogenesis without the additional effects of other cells (Udagawa et al., 1999). Because of the difficulties in isolating bone marrow cells from osteopetrotic bones, most studies involving osteopetrotic animals have used spleen cells as a source of haematopoietic precursors (Takahashi et al., 1991). Spleen is one of the secondary lymphoid organs, which contains numerous red blood cells, lymphocytes, macrophages and monocytes that can differentiate into osteoclasts given the proper stimulation.

In this thesis, only stroma-free differentiation cultures of osteoclasts were performed, thus initial experiments were set up in order to establish the optimal conditions for differentiation, which can vary according to different serum batches, mouse strain and source/activity of M-CSF and RANKL. Cell plating density experiments and dose-response analyses for M-CSF and RANKL identified the ideal parameters for obtaining functional. Moreover, TGF-β dose response experiments showed a dose-dependent increase in both osteoclasts number and size, consistent with published data (Fuller et al., 2000). However, although it has been reported that TNF-α could substitute for RANKL in supporting osteoclast differentiation in the presence of M-CSF, only very few TRAP positive multinucleated cells were generated at the highest concentration in the absence of RANKL. Thus, it is likely that under our experimental conditions, some RANKL is required to observe any TNF-α effects (Lam et al., 2000; Zhang et al., 2001b).

### 5.7.2 *In vitro* studies of *c-fos* knockout mice

#### 5.7.2.1 Osteoclast precursors lacking c-Fos have a limited potential to differentiate into functional osteoclasts

To date, all *in vitro* differentiation assays for studying the mechanism of the osteoclastogenesis deficiency in osteopetrotic animals have used spleen cells as the source of haematopoietic precursors, because of the difficulty in obtaining sufficient numbers of bone marrow precursors. Clearly, the splenic precursors have proven to be very valuable in understanding the role of c-Fos in osteoclast differentiation (Matsuo et al., 2000; Takayanagi et al., 2002b), however, as it has been hypothesised in this thesis, it is possible that the monocyte/macrophage precursors isolated from bone marrow are different from that of the spleen. As described before, RANKL and M-CSF are essential and sufficient for osteoclast formation. RANKL was first identified as a product of activated T cells, and T cells also produce M-CSF and other cytokines that stimulate osteoclastogenesis, such as IL-1, IL-6, and TNF-α, which play an important role in pathological bone loss (Theill et al., 2002). However, no osteoclasts, but only macrophages are formed in the spleen, even under inflammatory conditions, even though these two cell types are derived from the same monocyte/macrophage precursors, and abundant T cells are present in the spleen. Thus, the absence of osteoclastogenesis in spleen tissues suggests that the local spleen microenvironment is different from that in the bone marrow, and/or different precursor populations or precursors at different differentiation stages are present in the spleen versus the bone marrow.

In order to study the effect of the absence of c-Fos on osteoclastogenesis from precursors derived from different sources, I systematically compared the osteoclastic potential of cells derived from bone marrow and spleen tissues at different ages of c-Fos mutant and wild-type mice. Surprisingly, several TRAP positive multinucleated cells were formed from newborn mutant bone marrow cells, and these cells formed resorption pits as well, although the number of TRAP-positive multinucleated cells and pits were much less than that from the wild-type bone marrow and the formation time was much delayed (10 days). Nevertheless, these data showed for the first time that bone marrow precursors from newborn mice lacking c-Fos have a limited potential to differentiate into mature, functional osteoclasts. On

the other hand, spleen cells from the mutant newborn mice were unable to generate TRAP positive multinucleated cells, supporting the hypothesis that the M-CSF-dependent mononuclear precursors derived from spleen are different from bone marrow precursors.

During haematopoiesis in bone marrow, granulocyte-monocyte progenitors differentiate into promonocytes, which leave the bone marrow and enter the bloodstream. While circulating in the bloodstream, they further differentiate into monocytes, and then migrate into the various tissues and differentiate into specific tissue macrophages. Thus, the resident bone marrow macrophages are different from those in the spleen, skin and brain, which are specifically designated as Kupffer cells, dendritic cells and microglia, respectively, and each of these populations have different functions specific to each of those tissues. Thus, in the case of c-Fos deficiency, one interpretation is that early stage osteoclast progenitors in bone marrow have an intrinsic ability, at least in part, to differentiate into osteoclasts, while those in spleen couldn't. The possible mechanisms for this are discussed below in section 5.7.4. Alternatively, another explanation for the inability of newborn mutant spleen cells to form osteoclasts *in vitro* is that the number of progenitors in the mutant spleen is too low to form osteoclasts, compared to those in the bone marrow. Indeed, it was shown in wild-type spleens (*e.g.* Fig.4.2.1), where, compared to bone marrow cells, ten-fold more spleen cells were needed for osteoclast formation.

The other significant observation was that the osteoclastic potential in precursors lacking c-Fos was age-dependent. While newborn knockout bone marrow cells could clearly form multinucleated osteoclasts, only a few TRAP positive mononuclear cells were generated from 2-3 week old mutant bone marrow precursors, but they were nevertheless functional. In contrast, no TRAP positive cells or resorption pits were induced from c-Fos mutant adult bone marrow culture or from mutant spleen cells. These results suggest that the potential of c-Fos knockout bone marrow precursors to differentiate into later stage osteoclasts is diminished in adult animals. These data therefore provide a possible explanation for the histological observation that the osteopetrosis in c-Fos knockouts becomes progressively worse with age, with a clear evidence of a partial bone marrow space in embryonic and newborn animals, in contrast to older animals which are heavily osteopetrotic (Fig.4.2.2). The reasons for this are not entirely clear. In embryos and young growing mice, bone

formation and remodelling occur at a rapid rate, which might lead to the active and continuous generation of osteoclast progenitors. In contrast, bone remodelling is significantly reduced in adults and consequently, osteoclast progenitors may not be required at a high frequency and their turnover is reduced. Therefore, in the absence of c-Fos, the very low number of early progenitors would result in the inability to form osteoclasts. The diminished differentiation ability of precursors in the mutant mice is also consistent with the previous observations that transplantation of wild type bone marrow cells rescued the osteopetrosis in newborn c-Fos knockout mice when active bone remodelling was still occurring, but couldn't rescue the osteopetrosis in adult mutant mice (Okada et al., 1994).

An unexpected observation was that the number of TRAP positive multinucleated cells induced from c-Fos heterozygous precursors (c-Fos+/-) was significantly decreased than that from the wild-type precursors (c-Fos+/+). This occurred in both bone marrow and spleen cultures from newborns to the adults. This has not been reported before, and suggests that *c-fos* affects osteoclast formation in a gene dose-dependent manner. This is similar to the situation with NFκB, where Iotsova et al. reported that the number of osteoclasts was slightly reduced in single NFκB knockout mice, $p50^{-/+}p52^{-/-}$, and osteoclast differentiation was arrested before fusion in $p50^{-/-}p52^{-/+}$ mice which contained only mononuclear immature osteoclasts but exhibited a normal bone phenotype (Iotsova et al., 1997). Although the differentiation potential was significantly reduced in c-Fos heterozygous precursors *in vitro*, the mice developed a normal bone marrow cavity, which suggests that there are mechanism(s) *in vivo* that could compensate for the deficiency of c-Fos.

Although I showed in this thesis that TRAP-positive cells could be induced from newborn and young *c-fos* mutant bone marrow cells *in vitro*, it is clear that there are no TRAP-positive cells present *in vivo* at any age (Grigoriadis et al., 1994). This apparent inconsistency could be explained in several ways. First, although the *in situ* hybridisation results and previously published data showed that osteoblasts are capable of supporting osteoclast differentiation (Grigoriadis et al., 1994), it is not clear how efficient this process is *in vivo* vs *in vitro*. Other cell types, such as lymphocytes, and many systemic and local factors, which play important roles in osteoclast formation, are not present *in vitro*, but are present *in vivo*, where the balance of stimulatory and inhibitory factors may be altered in the absence of *c-fos*. Also, the *in*

*vitro* system, which uses maximal concentrations of purified RANKL and M-CSF proteins, may be more efficient in inducing osteoclast differentiation from *c-fos* -/- precursors than in an *in vivo* environment where the "real" concentrations are not known. Second, Udagawa et al. reported that c-Fos affects the proliferation of osteoclast precursors (Udagawa et al., 1996). One cannot rule out, therefore, that cell proliferation in the relatively pure cell populations and high plating cell densities used *in vitro* might not accurately reflect the proliferation of similar populations *in vivo*. Third, although there are no identifiable TRAP positive cells in c-*fos* mutant bones *in vivo*, the current hypothesis is that the partial resorption which occurs in these mice is performed by the mononuclear MMP-9 positive cells, which are probably osteoclast precursors. It is possible that *in vitro*, a small proportion of these cells may progress further in the osteoclast lineage and express subsequent markers such as TRAP and CTR. Finally, it should be noted that with the increasing number of knockout animals being generated for many genes, that there are often slight discrepancies in cell behaviour in *in vivo* vs *ex vivo/in vitro* cultures, which yield important information regarding gene function, rather than being interpreted as just *in vitro* artifact. Regarding osteoclast biology, the recent analysis of osteoclast differentiation in JNK1 knockout mice *in vivo* vs *in vitro* provides one such example, although there are several others concerning other AP-1 genes (David et al., 2002).

Taken together, the *in vitro* data have provided valuable novel information in understanding the role of *c-fos* regulates osteoclast formation. Clearly, osteoclasts can form in the absence of c-Fos, although at very low levels, and therefore, the existing notion that c-Fos is an essential gene for osteoclast differentiation, may need to be re-evaluated.

### 5.7.2.2 Osteoclastic marker genes are expressed in newborn and young *c-fos* knockout bone marrow cultures

To verify the *in vitro* results using molecular markers, and to try and investigate a possible mechanism to explain the low levels of osteoclast differentiation in the absence of c-Fos, detailed expression analyses were performed. Consistent with tissue culture results, RT-PCR analysis showed that the osteoclast markers TRAP and, most importantly, CTR, were expressed in newborn mutant bone marrow cultures, although the expression levels were much lower than the wild type, which was

expected due to their reduced numbers. However, the RT-PCR data for TRAP was not always consistent, showing some expression in adult mutant cultures, which is inconsistent with the absence of TRAP staining *in vitro*. Moreover, TRAP mRNA was detected even before differentiation in all cultures, which suggests that for measuring TRAP expression, RT-PCR may not be so reliable, or alternatively, in spite of transcription, there was no protein produced or the protein level was too low to be detected. On the other hand, CTR is the most specific marker for mature osteoclasts in bone (Hattersley and Chambers, 1989a), and CTR expression pattern paralleled exactly the appearance of osteoclasts *in vitro*. Unfortunately, CTR expression was not detected in mutant 2-3 weeks old cultures which formed TRAP positive mononuclear cells that formed pits. However, this was not surprising, as it is unlikely that CTR message could be detected from the very few TRAP positive mononuclear cells that were present in these cultures. Thus, these data definitely confirmed at the molecular level that mature osteoclasts were generated in the absence of c-Fos.

As expected, *RANK* mRNA was expressed in c-Fos mutant cells, although the level was much lower than in the wild type, which confirms the *in situ* hybridisation results shown in Chapter 3, and is consistent with published data (Matsuo et al., 2000). Additionally, the expression level was upregulated after differentiation both in the wild type and mutant cultures, which is consistent with previous experiments that M-CSF stimulates RANK expression on early-stage osteoclast precursors (Arai et al., 1999). As shown before, precursors isolated from c-Fos mutant spleen have less differentiation potential *in vitro* than those from bone marrow, however, paradoxically, the RT-PCR data showed higher RANK expression in mutant spleen cultures than in bone marrow. Although RANK is expressed primarily on osteoclastic cell lineage in bone, it is also expressed in T and B cells that are abundant in spleen (Anderson et al., 1997). Besides developing osteopetrosis, RANK knockout mice also lack peripheral lymph nodes and had defective B cell and T cell maturation (Li et al., 2000). Therefore most cells expressing RANK in spleen cultures could be lymphocytes, which could explain the RT-PCR results.

The mature macrophage marker, F4/80, was highly expressed in the mutant cultures, which correlates well with the *in vivo* expression pattern, and further supports the notion that bipotential precursors differentiated into the macrophage lineage in the absence of c-Fos. In contrast to the *in situ* expression data, there was no differ-

ence in *c-fms* expression, which is present on both osteoclasts and macrophages, between mutant and wild-type cultures at all ages tested. However, high levels of c-*fms* expression could perhaps be expected in the *in vitro* cultures, as the cells are selected in the presence of maximal doses of M-CSF, thus any differences between mutant and wild-type cultures may not be evident.

Taken together, semi-quantitative RT-PCR analysis of osteoclast and macrophage genes further confirmed that M-CSF and RANKL responsive progenitors are present in c-Fos mutant mice, and that these cells have a limited potential to differentiate into mature osteoclasts in an age-dependent manner.

### 5.7.2.3 Altered expression of signalling molecules in *c-fos* knockout cultures

During osteoclast differentiation, RANKL binds to RANK, the receptor on osteoclast precursors, which mediates signal transduction via TNF receptor-associated factor (TRAF) proteins, especially TRAF-6 (Naito et al., 1999; Wong et al., 1998). TRAF-6 knockout mice developed osteopetrosis due to the defect in osteoclast differentiation and activation (Lomaga et al., 1999; Naito et al., 1999). In the experiments with c-*fos* knockout mice, semi-quantitative RT-PCR showed that TRAF-6 expression was upregulated after differentiation in wild type cultures, while in mutant cultures, the expression remained unchanged after differentiation. This is consistent with low level of RANK expression in the same cells, and implies that the signalling pathway downstream of RANKL/RANK is attenuated in the absence of c-Fos.

With respect to other c-Fos-related genes, some interesting observations were made. Fra-1 is a transcriptional target of c-Fos in osteoclast progenitors, and overexpression of *fra*-1 rescued the differentiation of *c-fos* deficient osteoclast precursors both *in vivo* and *in vitro* (Fleischmann et al., 2000; Matsuo et al., 2000). Semi-quantitative RT-PCR analysis of *fra-1* expression in osteoclast cultures at different ages showed upregulation of *fra-1* expression after differentiation in the wild-type cultures, which coincides with osteoclast formation. Interestingly, although the expression level was low before differentiation, *fra-1* mRNA was strongly expressed in mutant newborn and 2-3 week old bone marrow cultures after differentiation, and the level appeared approximately two fold higher than the wild-type, although this was not quantified by densitometry. This increase could provide one possible expla-

nation for the osteoclastogenesis which occurred in these mutant cultures. Matsuo et al. reported that RANKL induces transcription of *fra-1* in a c-Fos-dependent manner (Matsuo et al., 2000). In their studies, *fra-1* expression in c-Fos-/- spleen cells was lower than in the wild type, and no induction was observed 0.5-2 hours after RANKL treatment. Similarly in the experiments shown here, *fra-1* expression was not upregulated in all the mutant spleen cultures, but also not in the adult bone marrow culture after differentiation. The fact that TRAP positive multinucleated and mononuclear cells were only formed in newborn and 2-3 weeks old mutant bone marrow culture, where *fra-1* was strongly induced by RANKL, suggests that in mutant progenitors from young mice, *fra*-1 is upregulated by some unknown, c-Fos-independent mechanism in response to osteoclastogenic stimuli, perhaps to compensate the deficiency of c-Fos. These results also suggest that Fra-1 may support osteoclast differentiation by a mechanism different from c-Fos, which is consistent with previous experiments that *fra-1* knockout mice developed normal bone structure with apparently normal numbers of TRAP positive osteoclasts present (Schreiber et al., 2000).

Fra-2 is another Fos family protein. In fibroblasts, expression of Fra-2 after growth stimulation is later than that of c-Fos, suggesting that it's a transcriptional target of c-Fos in this cell type (Suzuki et al., 1991), although this has not been shown directly. The reasons why this c-Fos-related gene has become interesting with respect to osteoclasts, is that it has recently been suggested that *fra-2* is a negative regulator of osteoclastogenesis. Recent preliminary data from Wagner and co-workers have demonstrated that *fra-2* knockout mice have an increased number of osteoclasts and developed osteoporosis (Karsenty and Wagner, 2002). In the experiments with *c-fos* knockout cells shown here, RT-PCR analysis of *fra-2* showed expression in bone marrow and spleen cultures both before and after differentiation, and there was no apparent differences between the wild-type and knockout cultures, which suggests that the regulation of *fra-2* transcription in osteoclast progenitors may be c-Fos-independent.

IFN-β has recently been identified as an inhibitor of osteoclastogenesis, playing an important role in c-Fos-mediated osteoclast differentiation. It is expressed and secreted by the osteoclast itself. Mice lacking IFN-β receptors developed osteoporosis with increased osteoclastic bone resorption, and IFN-β knockout mice had a

similar defect (Takayanagi et al., 2002b). In osteoclast precursors, RANKL induces the expression of IFN-β in a c-Fos-dependent manner, as the induction was absent in c-Fos deficient splenocytes. IFN-β then inhibits osteoclast differentiation by interfering with the RANKL-induced expression of c-Fos (Takayanagi et al., 2002b), which represents an important negative feedback regulation for osteoclastogenesis. Thus, *IFN-β* is both a c-Fos target gene, and is involved in negatively regulating c-Fos. It was therefore an interesting candidate gene for investigating in the differentiation of osteoclasts in *c-fos* knockout mice. Surprisingly, however, semi-quantitative RT-PCR showed that *IFN-β* was expressed in newborn and 2-3 week old mutant cultures both before and after differentiation. It was also expressed in adult cultures after differentiation, although before differentiation the expression levels were only detectable in adult mutant precursors, which is consistent with the results of Takayanagi et al. (Takayanagi et al., 2002b). Since in their experiments, *IFN-β* induction was absent in c-Fos deficient splenocytes after 2 and 24 hours RANKL stimulation, our results suggests that in long term culture, some mechanisms may substitute for c-Fos in mediating RANKL-induced expression of IFN-β. One candidate could be Fra-1, although it is not yet known whether Fra-1 can regulate IFN-β transcription. In addition, the expression of *IFN-β* in mutant bone marrow cultures was similar to that of wild-type cultures, while in mutant spleen cultures the expression was lower than in wild-type cultures, which further supported one of the hypotheses in this thesis that the precursors in spleen are different from those in bone marrow, at least in their response to RANKL.

Taken together, semi-quantitative RT-PCR analysis confirmed the tissue culture results that mature osteoclasts were formed in c-Fos-mutant newborn bone marrow cultures. Alternative signalling pathways, particularly in younger animals, including upregulation of *fra-1*, may compensate for the deficiency of c-Fos.

#### 5.7.2.4 TGF-β and TNF-α can not fully rescue osteoclastogenesis in the absence of *c-fos*

RANKL-induced osteoclastogenesis is regulated by various hormones and growth factors. TGF-β is one of the growth factors that act directly on osteoclast precursors and strongly promoted their differentiation (Fuller et al., 2000). Interestingly, it has been reported that TGF-β might facilitate osteoclast differentiation

through its role as a macrophage deactivator. Antibodies against TGF-β significantly inhibited osteoclast formation *in vitro* (Fox et al., 2000; Kaneda et al., 2000). Thus, the effect of TGF-β on osteoclastogenesis in the absence of c-Fos was investigated.

Consistent with previous data (Fuller et al., 2000), TGF-β strongly stimulated osteoclast formation and survival in the presence of RANKL and M-CSF in wild-type bone marrow cultures, however, the effect of TGF-β on resorptive function was much less significant. The possible explanation is that the culture time was prolonged slightly (11 days) in order to obtain measurable and reliable levels of bone resorption from mutant precursors. Therefore, in the corresponding wild-type cultures, the percentage of resorption was very high even in the absence of TGF-β, which may conceal the stimulatory effect of TGF-β. Interestingly, the number of TRAP positive multinucleated and mononuclear cells generated from newborn c-Fos mutant bone marrow cultures and their function were significantly increased by TGF-β. In addition, the number of TRAP positive multinucleated cells induced from TGF-β-treated heterozygous cultures was similar to that of wild-type cultures, which suggests that TGF-β may also partially rescue the reduction in differentiation caused by the absence of one c-*fos* allele.

The molecular mechanisms by which TGF-β stimulates osteoclast differentiation and survival are still not clear. TGF-β signals through Smad-2 and 3, which form heteromeric complexes with the co-Smad, Smad-4. These complexes are translocated into the nucleus, and regulate the transcription of target genes in co-operation with other transcription factors, e.g. AP-1 family, coactivators and corepressors (ten Dijke et al., 2000). It was reported that TGF-β synergistically increased RANKL-induced translocation of *NFκB*, an important transcription factor for osteoclastogenesis, into nuclei in osteoclast progenitors (Kaneda et al., 2000). *In vitro* binding studies showed that both Smad3 and Smad4 bind all three Jun family members: c-Jun, JunB, and JunD (Liberati et al., 1999). It is well established that c-Fos heterodimerises with Jun proteins, mainly c-Jun, exerting transcription activities. Furthermore, c-Jun co-operates with c-Fos in osteoclastogenesis (Wang et al., 1995). Koseki et al. showed that although TGF-β had no effect on JNK activity, it enhanced JunB expression (Koseki et al., 2002), which is reported to affect c-Jun-mediated transactivation and transformation. Hence, in the absence of c-Fos, TGF-β may enhance osteoclast for-

mation through NFκB and c-Jun signalling but this remains to be investigated. 12-O-tetradecanoyl-13-acetate (TPA)-responsive gene promoter elements (TREs) are involved in the transcriptional responses of several genes to TGF-β. c-Jun and c-Fos, bind to TREs as an AP-1 complex and act together with Smad3 and Smad4 to activate transcription in response to TGF-β. However, Smad3 and Smad4 can also activate TGF-β-inducible transcription from the TRE in the absence of c-Jun and c-Fos (Zhang et al., 1998). This suggests that in the absence of *c-fos*, TGF-β may enhance osteoclast differentiation by some alternative mechanisms. However, TGF-β failed to rescue the defect of osteoclast formation in newborn spleen cultures, and in 2-3 week old and adult bone marrow and spleen cultures. Furthermore, although osteoclastogenesis from heterozygous precursors in adult cultures was enhanced after TGF-β treatment, it was still much lower than that from the wild type precursors.

In conclusion, the partial rescue effect of exogenous TGF-β in the absence of c-Fos is restricted to the early progenitors and is RANKL-dependent. Exogenous TGF-β can not fully substitute for c-Fos in osteoclastogenesis.

TNF-α is a pro-inflammatory cytokine, which plays an important role in pathological bone resorption. In addition to its stimulatory effects on RANKL-induced osteoclastogenesis (Lam et al., 2000), TNF-α has been reported to be able to induce osteoclast formation independently of RANKL (Azuma et al., 2000; Fuller et al., 2002). Under our experimental conditions, TNF-α failed to induce osteoclasts in the absence of RANKL, supporting other reports by Lam et al. that basal levels of RANKL are required to "prime" the precursors for osteoclastogenesis (Lam et al., 2000). Thus, I also investigated the effect of TNF-α on osteoclast differentiation in the absence of c-Fos.

As expected, TNF-α enhanced RANKL-induced osteoclast formation in wild-type cultures and this appeared to occur over a shorter time course. However, the number of TRAP positive multinucleated cells decreased dramatically in long-term TNF-α culture more rapidly than in wild-type cultures, suggesting either that TNF-α induces osteoclast apoptosis, or that osteoclasts undergo their normal programme of apoptosis earlier than wild-type cells since they form earlier. The function of TNF-α is mediated by two receptors: TNF receptor1 (TNF-R1) and 2 (TNF-R2). TNF-α binding to TNF-R1 leads to activation of NFκB, ERKs and JNK, which are

important signalling molecules for osteoclast formation (Zhang et al., 2001b). However, binding of TNF-α to TNF-R1 also induces the recruitment of FADD and caspase-8 which leads to apoptosis (Barkett and Gilmore, 1999). TNF-α didn't rescue the osteoclastogenesis defect in c-Fos deficient precursors from newborn and 2-3 week old mutant mice, but it nevertheless had a significant effect as many more TRAP positive mononuclear cells were generated in mutant bone marrow cultures, though the staining was faint. These results suggest that in the presence of RANKL, TNF-α may indeed enhance the differentiation of precursors towards the osteoclast pathway, however, in the absence of c-Fos, these cells fail to differentiate further into mature osteoclasts. This is consistent with previous experiments in which transgenic mice overexpressing TNF-α, which develop rheumatoid arthritis-like symptoms, were intercrossed with c-Fos knockout mice. The resulting TNF-α Tg/c-Fos-/- mice still developed osteopetrosis but with a non-destructive arthritis (Redlich et al., 2002), suggesting that exogenous TNF-α could not rescue the block in osteoclast differentiation *in vivo*.

The inability of TNF-α to rescue the defect of osteoclastogenesis in the absence of c-Fos is perhaps not surprising, since both TNF-α and RANKL belong to the TNF ligand superfamily, and their signalling pathways are considerably overlapping (Zhang et al., 2001b). Moreover, TRAF-6 is also critical for TNF-α-induced osteoclastogenesis, and osteoclast formation in the presence of M-CSF and TNF-α was abolished in spleen cell cultures derived from TRAF-6 deficient mice (Kaji et al., 2001). Together with the fact that TNF-α induces AP-1 activity (Brenner, nature 1989), the tissue culture results suggest that c-Fos is indispensable for TNF-α induced osteoclastogenesis.

Taken together, TGF-β and TNF-α stimulate RANKL-induced osteoclastogenesis in a c-Fos-dependent manner, and TGF-β can enhance osteoclast formation in early c-Fos mutant progenitors.

### 5.7.3 *In vitro* studies of *c-fos* transgenic mice

#### 5.7.3.1 Osteoclast precursors overexpressing c-Fos have an enhanced potential to differentiate into functional osteoclasts

The experiments described in this thesis also demonstrated a novel role for c-Fos as a positive regulator of osteoclast differentiation, using the c-Fos transgenic mice as a model system. Exogenous c-Fos may enhance osteoclastogenesis through several mechanisms: (1) exogenous c-Fos could increase the ratio of RANKL:OPG in local environment, hence exerting its effect indirectly via osteoblasts/stromal cells; (2) high levels of c-Fos in osteoclast precursors could substitute for the requirement of RANKL and M-CSF in osteoclast differentiation; (3) overexpression of c-Fos in osteoclast precursors could increase their responsiveness to osteotropic factors; and (4) exogenous c-Fos could prolong the survival of osteoclasts and their precursors.

It was important to check whether c-Fos affects osteoclast differentiation indirectly through osteoblasts/stromal cells, because osteoblastic cells are target cells for c-*fos* in these mice, leading to osteosarcoma formation (Grigoriadis et al., 1993). Osteoblasts from newborn mouse calvaria are widely used for supporting osteoclast generation *in vitro*. However, in the c-Fos transgenic mice, the expression of exogenous *c-fos* starts from 2-3 weeks after birth in bone tissues although primary cultures of stromal cells were never investigated (Grigoriadis et al., 1993). Additionally, previous experiments showed that osteoblasts of adult mouse calvarial bone expressed decreased level of RANKL and didn't readily support osteoclast formation (Kartsogiannis et al., 1999), whereas stromal cells from adult bone marrow were able to support osteoclast formation *in vitro* (Hattersley and Chambers, 1989c; Takahashi et al., 1988b; Udagawa et al., 1990).

Northern blot analysis of RANKL and OPG expression using RNA isolated from c-Fos transgenic and wild-type stromal cells, showed that stromal cells expressed very low basal levels of RANKL and OPG, which were increased and decreased, respectively, by treatment with $1,25(OH)_2D_3$ and dexamethasone, consistent with previous studies (Horwood et al., 1998; Udagawa et al., 1999). Furthermore, the levels of RANKL and OPG were similar in both transgenic and wild-type cells. Examining whether the c-Fos transgenic stromal cells actually expressed the transgene using transgene-specific primers, revealed that stromal cells from c-Fos transgenic

mice expressed very low levels of the transgene. These results suggest that it is unlikely that exogenous c-Fos affects osteoclastogenesis through osteoblastic cells.

The question of whether overexpression of c-Fos has direct effects on osteoclasts is still not well understood. As mentioned in the Introduction (section 1.7.3.2), previous data from our and Wagner's laboratories examining the osteoclasts within c-Fos transgenic tumours, and the generation of TRAP-c-*fos*LTR mice, provided circumstantial evidence supporting a direct role for c-Fos in stimulating osteoclasts (Grigoriadis et al., 1993; Wang et al., 1995; Beedles et al., 1999). An effect of c-*fos* has also been implicated in the differentiation of chick osteoclasts, as overexpression of c-Fos directly enhanced osteoclastogenesis *in vitro* (Miyauchi et al., 1994). In contrast, however, Owens et al. reported that infection of immortalised osteoclast/macrophage precursor cell lines and spleen cells with a c-Fos expressing retrovirus had no effects on osteoclast formation when cultured with stromal cells (Owens et al., 1999). Finally, Takayanagi et al. recently reported that bone marrow monocyte/macrophage precursors infected with a c-Fos retrovirus differentiated into TRAP positive cells in the absence of RANKL, although the efficiency was very low (7%) (Takayanagi et al., 2002a). The reasons for these inconsistencies are not very clear, but may be due to the different culture systems employed. In this thesis, the role of exogenous c-Fos was investigated in an *ex vivo* system, by examining M-CSF-dependent nonadherent bone marrow cultures from c-Fos transgenic mice compared to wild type littermates.

First, these experiments were feasible only after confirming that the c-*fos* transgene was expressed in M-CSF-dependent nonadherent bone marrow cells from c-Fos transgenic mice, which suggests that the transgene may act directly on osteoclast precursors. This was also consistent with the *in situ* hybridisation results that the transgene was expressed in TRAP positive mononuclear cells within the tumours and was downregulated after differentiation.

No TRAP positive cells were formed from c-Fos transgenic bone marrow precursors cultured with RANKL or M-CSF alone, suggesting that exogenous c-Fos is unable to substitute the requirement of RANKL and M-CSF signalling in osteoclast formation. Nevertheless, overexpression of c-*fos* had a clear effect on the responsiveness of osteoclast precursors to M-CSF and RANKL. The results clearly showed that the number of TRAP positive multinucleated cells was greater in c-*fos*-

overexpressing cells at all doses of M-CSF and RANKL tested, and this correlated well with resorptive activity.

Taken together, these results suggested that overexpression of *c-fos* indeed has the potential of promoting osteoclastogenesis by directly acting on osteoclast precursors and increasing their sensitivity to M-CSF and RANKL, independently of any effects on osteoblasts/stromal cells.

In view of the TGF-β and TNF-α effects discussed above, the possibility that high levels of c-Fos might have effects on TGF-β and TNF-α responsiveness was also investigated. Although TGF-β significantly increased the number of osteoclasts and stimulated resorption in all experiments, these stimulatory effects were similar in both c-Fos transgenic and wild type bone marrow cultures, suggesting exogenous c-Fos in osteoclast precursors has no effect on TGF-β responsiveness. Similarly, c-Fos transgenic precursors cultured with M-CSF and TNF-α gave rise to some TRAP positive mononuclear cells, but no multinucleated cells, suggesting that high levels of c-Fos had no effect on the responsiveness of precursors to TNF-α. Additionally, these results fit with the previous observation that, under our experimental conditions, TNF-α requires some additional RANKL in order to see its effects. It would therefore be interesting to see whether TNF-α, in the presence of RANKL, has different effects in the presence of exogenous c-Fos. Nevertheless, these data suggest that overexpression of c-Fos does not appear to alter TGF-β or TNF-α signalling.

Finally, the last possible function of c-Fos could be related to cell survival. Previous studies showed that c-Fos might have pro- and anti-apoptotic function depending on the cell type and the apoptotic stimulus. Wenzel et al. demonstrated that c-Fos is essential for light-induced apoptosis of retinal photoreceptors (Wenzel et al., 2000). Also, overexpression of c-Fos stimulated the apoptosis of retinal ganglion cells (Oshitari et al., 2002), and ectopic c-Fos induced apoptotic cell death in pro-B cells (Hu et al., 1996). On the other hand, c-Fos protected fibroblasts from UV induced apoptosis (Schreiber et al., 1995), and immature CD8(+)4(+) double-positive thymocytes from $Ca2^{+}$- and cAMP-induced apoptosis (Ivanov and Nikolic-Zugic, 1997). The TUNEL staining performed on osteoclast cultures following withdrawal of serum and growth factors showed that the number of apoptotic cells appeared to

be similar in both wild type and c-Fos transgenic cultures, suggesting that high levels of c-Fos had no effect on apoptosis in the osteoclast lineage.

Taken together, these studies demonstrated that ectopic c-Fos expression has direct effects on cells of the osteoclast lineage. Specifically, overexpression of c-Fos enhances RANKL-induced osteoclastogenesis directly by upregulating the responsiveness of osteoclast precursors to RANKL and M-CSF, but does not appear to play a role in modulating TGF-β and TNF-α signalling or cell survival.

### 5.7.3.2 Altered expression of osteoclastic markers and signalling molecules in *c-fos* transgenic cultures

Similarly to the molecular analyses performed using *c-fos* knockout cells, semi-quantitative RT-PCR analysis of osteoclastic marker genes was performed, which further confirmed that osteoclastogenesis was enhanced in osteoclast precursors overexpressing c-Fos. Expression of mature osteoclast specific marker genes, such as *CTR* and *cathepsin K*, was increased. The increased *cathepsin K* expression may also be responsible for the increased bone resorption. On the other hand, *TRAP* and c-*fms* expression levels were the same in both transgenic and wild-type cultures, which, as in the case of the knockout cultures described earlier, may not be surprising since this *in vitro* system high levels of RANKL and M-CSF restrict cells to the osteoclast and macrophage lineages. Indeed, almost all the cells in cultures after RANKL stimulation were TRAP positive, and the selection of M-CSF-dependent precursors ensures that all cells are expressing c-*fms*.

Interestingly, the expression of *RANK*, the receptor of RANKL, was significantly increased in c-Fos transgenic bone marrow cultures both before and after differentiation. The fact that M-CSF stimulates *RANK* expression on early-stage osteoclast precursors (Arai et al., 1999), together with the observation that *RANK* expression in M-CSF-dependent precursors isolated from c-Fos transgenic mice is higher than that from wild-type littermates, suggests that the high responsiveness of c-Fos transgenic precursors to M-CSF during osteoclastogenesis is at least partially due to the increased RANK expression after M-CSF stimulation. In addition, the increased RANK expression in c-Fos transgenic bone marrow cultures before and after differentiation could also explain the high responsiveness of c-Fos transgenic precursors to RANKL. Thus, more RANKL responsive cells present in the culture, or more recep-

tors present on precursors, would result in increased RANKL/RANK signalling. It has been reported that $RANK^-$ precursors differentiate into osteoclasts more efficiently than $RANK^+$ *cells in the presence of RANKL and M-CSF, as* $RANK^-$ *cells* have higher proliferative activity (Arai et al., 1999). However, in this experiment here, cells were not fractionated. $RANK^-$ and $RANK^+$ cells were not separated and therefore it was not possible to evaluate the consequences of overexpression of c-Fos from both cell populations.

TRAF-6 is the cytoplasmic adapter protein that mediates RANKL/RANK signalling in osteoclast differentiation and activation via activating NF-κB and JNK/AP-1 pathways (Lomaga et al., 1999; Naito et al., 1999). The expression of *TRAF-6* in c-Fos transgenic bone marrow cultures was higher than wild-type cultures even before RANKL treatment. These results suggest that exogenous c-Fos may potentiate osteoclast differentiation by inducing *TRAF-6* expression in osteoclast/macrophage precursors, and upregulating *TRAF-6* transcription upon RANKL and M-CSF stimulation.

As a target gene of c-Fos in osteoclast progenitors, *fra-1* expression was induced by exogenous c-Fos in precursors in the absence of RANKL similarly to *TRAF-6*. Moreover, *fra*-1 expression was much higher in transgenic cultures after differentiation than that in wild type cultures, which is consistent with the *in situ* hybridisation results in the transgenic tumours. Thus, it is likely that high levels of c-Fos may enhance osteoclastogenesis via inducing *fra-1* expression, at least in the context of this transgenic mouse line. Another c-Fos-related protein, Fra-2, which is proposed to be a negative regulator of osteoclastogenesis (Hoebertz et al, 2003), is also expressed in osteoclast precursors and mature osteoclasts generated *in vitro*, however, there were no differences in *fra-2* expression between wild-type and transgenic bone marrow cultures. Similarly, *IFN-β* expression in c-Fos transgenic bone marrow cultures was the same as wild type cultures. In osteoclast precursors, *IFN-β* expression was absent and became detectable after differentiation. As described above, *IFN-β* is both a c-Fos target gene and an inhibitor of osteoclastogenesis, thus, RANKL-induced c-Fos expression induces its own inhibitor (Takayanagi et al., 2002b). This negative feedback is important for controlling osteoclast differentiation and bone resorption. In addition, overexpression of c-Fos by virus infection rescued the inhibitory effect of IFN-β in osteoclast differentiation (Takayanagi et al., 2002b).

In the c-Fos transgenic mice, overexpression of c-Fos doesn't increase IFN-β expression which may be one of the mechanisms by which high c-Fos levels enhances osteoclast formation.

Thus, positive signalling downstream of RANKL/RANK, such as *TRAF-6* and *fra*-1 (see also below), and negative regulatory mechanisms, such as *IFN-β*, may both be involved in mediating the effect of the c-Fos transgene on osteoclastogenesis.

### 5.7.4 The relationship between *c-fos* levels, *fra-1* expression and osteoclast differentiation

Finally, the correlation between *fra*-1 expression and c-*fos* levels in both c-*fos* knockout and transgenic cultures prompted a further investigation of the short term response of *fra*-1 to RANKL in the different mutants. Previous experiments showed that RANKL induced *fra-1* expression in wild type M-CSF-dependent precursors within 1 hour, while no induction was observed in c-Fos deficient adult spleen precursors (Matsuo et al., 2000). Experiments in this thesis using c-Fos knockout cells from 2-3 week old bone marrow and spleen, confirmed that *fra-1* mRNA expression level was not upregulated after 1 hour of RANKL stimulation. This correlates well with the fact that these cell populations do not differentiate into multinucleated osteoclasts. Interestingly, however, in newborn cultures, which do have the potential to form osteoclasts in the absence of c-Fos, RANKL induced *fra-1* expression in c-Fos mutant bone marrow cultures, and the levels were even higher than in the wild-type cultures. In contrast, RANKL didn't induce *fra-1* expression in newborn spleen cells, which cannot form osteoclasts. The difference in *fra-1* induction between newborn and 2-3 week bone marrow suggests that, specifically in mutant bone marrow osteoclast precursors, *fra-1* expression is upregulated by some c-Fos-independent mechanisms, which is both age-dependent, and specific to bone marrow precursors. The absence of RANKL- induced *fra-1* expression in newborn mutant spleen cells further confirmed that precursors in spleen are different from that in bone marrow.

The relationship between c-*fos*, *fra*-1 and osteoclast differentiation was also consistent in c-Fos transgenic experiments. In bone marrow precursors overexpressing c-*fos*, the levels of RANKL-stimulated *fra-1* expression were higher than in wild type cells, and the induction occurred earlier and reached peak levels earlier. These

results clearly suggested that high levels of exogenous c-Fos augment RANKL-induced *fra-1* expression, and this could provide an explanation for the increased osteoclast formation seen in the transgenic cultures.

Taken together, the regulation of both basal and RANKL-induced *fra*-1 expression has paralleled very closely the levels of c-Fos in both the knockout as well as the transgenic cultures, in an age-dependent manner. The two exceptions are in the newborn and young c-Fos mutant bone marrow cultures, which is the only population which can form osteoclasts, and in which *fra*-1 expression appears to be independent of c-Fos. Moreover, the regulation of *fra*-1 has correlated well with the osteoclastogenic potential of bone marrow versus spleen-derived precursors.

# Chapter 6

# General discussion and conclusions

In vertebrates, the integrity of the skeleton depends on bone remodelling in which osteoclastic bone resorption is followed by osteoblastic bone formation. Dysfunction of, and imbalance between osteoblasts and osteoclasts lead to bone metabolic diseases such as osteoporosis, in which bone resorption exceeds bone formation, or osteopetrosis, in which bone formation exceeds bone resorption (Karsenty and Wagner, 2002). A detailed understanding of bone cell biology may therefore provide fundamental insights into the etiology and treatment of bone diseases.

Osteoclasts are multinucleated cells responsible for bone degradation. Osteoclastic bone resorption is important for both bone development and bone remodelling. The differentiation and activation of osteoclasts are tightly regulated by cellular and hormonal factors *in vivo*. Transcription factors are involved in the early stages of osteoclast differentiation, regulating the development of bipotential precursors and commitment of precursors to the osteoclast lineage, and the role of transcription factors in osteoclastogenesis has been elucidated by targeted gene deletion and overexpression in mice (Karsenty and Wagner, 2002; Wagner and Karsenty, 2001).

Among the transcription factors identified so far, c-Fos is unique in that it appears to act at the branch point where osteoclast and macrophage differentiation diverge. Mice lacking c-Fos developed osteopetrosis due to complete absence of functional osteoclasts and their immediate precursors, however, the number of macrophages in mutant bone marrow was increased (Grigoriadis et al., 1994). Therefore, c-Fos knockout mice provide an ideal model for studying the biology of osteoclast. On the other hand, c-Fos is a proto-oncogene, which belongs to the AP-1 transcription family. Overexpression of c-Fos resulted in osteosarcomas with active bone remodelling. However, tumour formation was almost absent in c-Fos knockout mice expressing the c-Fos transgene (Wang et al., 1995), which suggests that high levels of c-Fos may enhance osteoclast formation which plays an important role in tumorigenesis. The aims of this project therefore, were to investigate the mechanisms by which c-Fos controls osteoclastogenesis, and how osteoclasts are involved in tumour development.

Determining where the block in osteoclast-macrophage development occurs is an important step in discovering the function of *c-fos* in the differentiation of this lineage. Both *in vivo* and *in vitro* experiments showed that early M-CSF- and RANKL-responsive osteoclast progenitors are present in c-Fos mutant long bones.

Mapping the expression of osteoclast and macrophage specific markers shown in chapter 3 showed that in the osteoclast differentiation pathway, c-Fos functions at a point very close to NF-κB, downstream of PU.1, M-CSF and RANK, and upstream of Mitf.

Pathogenic defects in osteoclastogenesis may be caused by defects intrinsic either to the osteoclast lineage or to the stromal cell lineage. Osteoblasts or bone marrow stromal cells are required for osteoclast formation and activation *in vivo*. The production of RANKL and OPG, the two key factors for osteoclastogenesis, by mutant osteoblasts was confirmed in chapter 3. Although the ratio of RANKL:OPG in c-Fos knockout bone marrow compared to wild-type was not determined, the high expression and the apparently normal hormonal *regulation suggested that it is unlikely* that altered stromal cell expression of RANKL and OPG can explain the osteoclast defect.

Most *in vitro* differentiation assays studying the mechanism of the osteoclastogenesis defect in osteopetrotic animals have used spleen cells as the source of haematopoietic precursors. This is based on two main reasons. First, due to the drastically reduced bone marrow space, it was always thought to be very difficult and impractical to isolate large numbers of bone marrow cells to study osteoclast differentiation. Second, in all osteopetrotic animals, extramedullary haematopoiesis occurs in the spleen, in which the production and differentiation of precursors is normal – this was proven experimentally in the c-Fos knockout mice (Okada et al., 1994). However, it was hypothesised in this thesis, and demonstrated in chapter 4, that M-CSF-dependent precursors derived from bone marrow are different from that of spleen, at least with respect to their absolute dependence on c-Fos for differentiation. This new concept may help to further understand the nature of osteoclast precursors and their distribution in the body. The bone marrow culture system developed in this project provides a new model for studying the differentiation potential of authentic osteoclast precursors from osteopetrotic mice *in vitro*, and to investigate the factors which might rescue the differentiation block in these precursors. However, this model is not perfect. Even though M-CSF-dependent cells were isolated over a 2-day period, stromal cell contamination couldn't be ruled out, and this would apply more to the mutant cultures as there is a higher degree of unresorbed cartilage and bone present. Thus, the production of local factors other than RANKL or OPG, or cell-cell interac-

tions from this potential stromal cell contamination might affect cell proliferation and fusion of osteoclast precursors. Future experiments to address this are to perform longer time M-CSF incubation, or use other techniques such as MACS sorting for c-*fms* positive cells which will yield a stromal-free precursor population. This could also be complimented by examining the expression of stromal cell markers following purification.

Nevertheless, it is first shown here that early stage osteoclast progenitors from mutant newborn bone marrow have limited potential to differentiate into functional mature osteoclasts in the absence of c-Fos, which is diminished in adults. This is the first report implying that c-Fos may not necessarily be an essential gene for osteoclast differentiation, and that there might be c-Fos-independent ways of generating osteoclasts. This is consistent with previous results that bone marrow transplantation can fully rescue the osteopetrosis in newborn mutant mice, but not in adults (Okada et al., 1994). Moreover, the data shown in this thesis are supported by recent ongoing experiments in our laboratory using Embryonic Stem (ES) cells to generate osteoclasts. The results so far have shown that ES cells lacking c-Fos have the capacity to differentiate into osteoclasts with less efficiency than wild-type ES cells, but to an apparently greater extent than knockout newborn bone marrow cultures. Thus, these data further confirm that c-Fos is not essential for osteoclast differentiation, and suggested that the differentiation potential of precursors in the absence of c-Fos is decreased during development.

The mechanisms that substitute for c-Fos in newborn precursor differentiation are not clear. Presumably, other AP-1 family members may be involved, and Fra-1 is clearly one of them, for all the reasons discussed extensively in this thesis. This is supported by data in this thesis, since *fra*-1 levels were elevated in all instances where knockout cells gave rise to TRAP positive osteoclasts, and were diminished in knockout cells which failed to form osteoclasts. However, this possibility must be viewed carefully, since the expression of *fra-1* is not restricted to the osteoclast lineage. As other cell types, such as macrophages can also express *fra-1*, high levels of *fra-1* expression detected both *in vivo* and *in vitro* may reflect the higher number of macrophages present in mutant bone marrow. Moreover, loss-of-function experiments, have shown that *fra-1* knockout mice developed normal bone structure with TRAP positive osteoclasts present (Schreiber et al., 2000). Thus, the mechanisms

by which *fra-1* is upregulated, and roles of *fra-1* in osteoclast differentiation in the absence of c-Fos need further investigation.

The Jun family members, especially *c-jun*, may also play a role in osteoclast differentiation, although Matsuo et al. reported that none of the Jun proteins rescued differentiation in c-Fos knockout spleen cells (Matsuo et al., 2000). During osteoclastogenesis, binding of RANKL to RANK activates signalling transduction pathways, such as NF-κB and JNK, via TRAF-6. JNK is one of the MAP kinases, which phosphorylates the N-terminal domain of Jun and modulates the transcriptional activity of AP-1. JNK contains at least 10 different isoforms encoded by three different genes: *Jnk1*, *Jnk2* and *Jnk3*. Osteoclastogenesis is impaired in haematopoietic cells isolated from mice lacking JNK1, or c-Jun, or expressing a mutated form of c-Jun that cannot be phosphorylated by JNKs (David et al., 2002), which suggest that JNK1-dependent c-Jun phosphorylation in response to RANKL is essential for efficient osteoclast formation. The level and function of c-Jun and JNK in precursors lacking c-Fos was not analysed in this project, and this represents an interesting area of future investigation.

Finally, another candidate for substituting the function of c-Fos in osteoclast differentiation is NFATc1, which was recently reported to be a new c-Fos target. NFATc1, a member of the NFAT (nuclear factor of activated T cells) family, is most strongly induced following RANKL stimulation, and it's expression is dependent on both the TRAF6 and c-Fos pathways (Takayanagi et al., 2002). Indeed, precursors overexpressing NFATc1 efficiently differentiated into osteoclasts even in the absence of RANKL. The expression of NFATc1 in c-Fos mutant haematopoietic cells derived from knockout mice of different ages, and whether overexpression of NFATc1 could rescue or enhance the c-Fos effect, would therefore be extremely interesting future experiments to see if this transcription factor could explain why osteoclasts can form in the absence of c-Fos.

Additional cytokines which are known to stimulate osteoclastogenesis were added to c-Fos mutant cultures in attempt to rescue the differentiation block. Exogenous TGF-β could not substitute completely for c-Fos, but it did augment osteoclastogenesis in newborn mutant bone marrow precursors, which suggests that TGF-β may act through both c-Fos-dependent and independent pathways during osteoclastogenesis. Previous studies have shown that endogenous TGF-β is essential for os-

teoclastogenesis, since an anti-TGF-β antibody abrogated RANKL- and TNF-α-induced osteoclast formation *in vitro* (Fox et al., 2000; Kaneda et al., 2000). The effect of overexpression of TGF-β in osteoclast precursors on osteoclastogenesis in the absence of c-Fos could be investigated by virus infection or by transgenic mice. TNF-α, on the other hand, failed to rescue the defect of osteoclastogenesis in c-Fos deficient precursors, which may be due to its pro-apoptotic effect (Barkett and Gilmore, 1999). Since TGF-β prolongs the survival of osteoclasts (Fuller et al., 2000), TNF-α combined with TGF-β may be more efficient in rescuing the defect of osteoclastogenesis in the absence of c-Fos.

With regard to overexpression of c-Fos, only previous circumstantial data suggested that high levels of c-Fos may enhance osteoclast formation directly. Here, it was proved for the first time by both *in vivo* and *in vitro* studies that overexpression of c-Fos clearly has effects on osteoclast differentiation. However, the mechanisms by which overexpression of c-Fos upregulates the responsiveness of osteoclast precursors to RANKL and M-CSF are not clear. The survival of osteoclasts and their precursors was apparently not affected by the transgene and may be ruled out as a possible mechanism. However, the effect of exogenous c-Fos on cell proliferation was not investigated, and experiments such as BrdU staining should be performed in order to address this question. The evidence also suggested that signals downstream of the RANKL/RANK pathway, such as TRAF-6 and *fra-1*, and negative regulatory factors, such as IFN-β, may be involved in the effect of c-Fos transgene on osteoclastogenesis and these studies remain to be performed.

Finally, in the context of oncogenesis, experiments showed that osteoclasts are important for bone tumorigenesis (Clohisy and Ramnaraine, 1998; Wang et al., 1995). Zhang et al. reported that injection of cancer cells intratibially and subcutaneously in mice induced tumorigenesis. Administration of OPG completely prevented the establishment of mixed osteolytic/osteoblastic tibial tumours, but had no effect on subcutaneous tumour growth (Zhang et al., 2001). In c-Fos-induced osteosarcomas, numerous multinucleated osteoclasts and macrophages are present. Especially interesting was the observation that osteoclastic marker genes are also expressed in fibroblastic areas of osteosarcomas not containing any bone, suggesting that exogenous c-Fos had the capacity to induce ectopic osteoclast formation. Transformed fibroblasts and/or pre-osteoblasts in these areas express high levels of RANKL and OPG, which

could support osteoclast formation from macrophages, that are present in high numbers in the tumours. In terms of target genes, Fra-1 emerged as a clear target which might be important for c-Fos-induced osteoclastogenesis, at least in the context of osteosarcoma formation and tumour remodelling. It will therefore be important to check the *in situ* expression of the other c-Fos target genes in a systematic manner. Some interesting experiments remain in order to address the role of osteoclasts, and of Fra-1, on the progression of c-Fos-induced osteosarcomas. For example, it would be predicted from these studies that treatment of c-Fos transgenic mice with osteoclast inhibitors, such as OPG, would not inhibit tumour initiation, but progression. Also, whether tumour formation is altered in c-Fos transgenic mice crossed with Fra-1 knockout mice, which have osteoclasts, would provide genetic evidence on the role of Fra-1 in osteoclast differentiation in c-Fos induced tumours.

In conclusion, c-Fos affects osteoclast differentiation in a gene-dose dependent manner, and the effects are most likely mediated by its target gene *fra-1*. The different differentiation potential between bone marrow and splenic haematopoietic progenitors in the absence of c-Fos, provides further information on understanding the nature of osteoclast precursors, and suggests a need to re-evaluate the role of c-Fos in osteoclast differentiation. Furthermore, the culture system employed in this thesis provides a useful tool for studying osteoclastogenesis in osteopetrotic animals. Finally, the enhancement of overexpression of *c-fos* on osteoclastogenesis, together with the ectopic osteoclast formation in c-Fos-induced osteosarcomas, aids in understanding bone tumour progression, and is useful for identifying new approaches for treatment of primary bone tumours.

# Chapter 7

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